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(54) **Novel amyloid precursor proteins and methods of using same**

Neue Amyloid-Precursor-Proteine und Verfahren zur deren Verwendung

Nouveaux précurseurs de protéines amyloides et méthodes les utilisant

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- **SCIENCE vol. 248, 1990, pages 492 - 495 S.S. SISODIA ET AL.; 'Evidence that beta-amyloid protein in Alzheimer's Disease is not derived by normal processing'**
- **JOURNAL OF BIOLOGICAL CHEMISTRY vol. 267, 1992, pages 25602 - 25608 S.R. SAHASRABUDHE ET AL.; 'Release of amino-terminal fragments from amyloid precursor protein reporter and mutated derivatives in cultured cells'**

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Description

BACKGROUND OF THE INVENTION

[0001] Throughout this application various references are referred to within parentheses. Disclosures of these publications in their entirety are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citations for these references may be found at the end of this application, immediately preceding the claims.

[0002] Abnormal accumulation of extracellular amyloid in plaques and cerebrovascular deposits are characteristic in the brains of individuals suffering from Alzheimer's disease (AD) and Down's Syndrome (Glenner and Wong, BBRC, 120:885-890, 1984; Glenner & Wong, BBRC, 120:1131-1153, 1984). The amyloid deposited in these lesions, referred to as beta amyloid peptide (BAP), is a poorly soluble, self-aggregating, 39 to 43 amino acid (aa) protein which is derived via proteolytic cleavage from a larger amyloid precursor protein (APP) (Kang et al., Nature 325:733-736, 1987) BAP also is thought to be neurotoxic (Yankner et al., Science 245:417-420, 1990). APP is expressed as an integral transmembrane protein (Dyrks et al., EMBO. J., 7:949-957, 1989) and is normally proteolytically cleaved by "secretase" (Sisodia et al., Science, 248:492-495, 1990; Esch et al., Science, 248:1122-1124) between BAP-16K (lysine) and -17L (leucine). Cleavage at this site therefore precludes amyloidogenesis (Palmert et al., BBRC, 156:432-437, 1988) and results in release of the amino-terminal APP fragment which is secreted into tissue culture medium (Sisodia et al., ibid, Esch, et al., ibid). Three major isoforms of APP (APP-695, APP-751 and APP-770 amino acids) are derived by alternative splicing (Ponte, et al., Nature 331:525-527, 1988; Kitaguchi et al., Nature 331:530-532, 1988; and Tanzi, et al., Nature 331:528-530, 1988), are expressed as integral transmembrane proteins (Kang et al., Nature 325:733-736, 1987; Dyrks et al., EMBO J. 7:949-957, 1988).

[0003] Even though both APP-770 and -751 isoforms contain a protease inhibitor domain, it is the secreted portion of APP-751 (also known as Protease Nexin II (Van Nostrand et al., Science, 248:745-748, 1990) which is thought to be involved in cell adhesion (Schubert et al., Neuron, 3:689-694, 1989), remodeling during development, coagulation (Smith et al., Science, 248:1126-1128, 1990) and wound repair.

[0004] Although the mechanisms underlying abnormal proteolytic processes which result in BAP extraction from APP are poorly understood, it is thought to be central to the pathogenesis (Selkoe, Neuron, 6:487-498, 1991; Isiura, J. Neurochem. 56:363-369, 1991) and memory loss (Flood, et al., Proc. Natl. Acad. Sci. 88:3363-3366, 1991) associated with Alzheimer's Disease.

[0005] Based on the observations that (a) amyloid plaques develop in AD brains, (b) a major component of plaques is BAP, (c) BAP is generated by proteolytic cleavage of APP protein, (d) mRNA levels of specific APP isoforms increase in AD suggesting that more APP protein is expressed, (e) APP point mutations which are thought to possibly after normal processing have been identified in Familial AD (FAD) and "Dutch" disease, (f) injection of BAP into the brains of rodents both form lesions reminiscent of plaque pathology and result in memory deficits, and (g) the detection of plaque-like amyloid deposits in the brains of transgenic mice expressing human APP, it is important to understand how APP is processed to generate BAP.

SUMMARY OF THE INVENTION

[0006] This invention provides novel nucleic acid molecules which encode amyloid precursor muteins and the polypeptides encoded therefrom. Also provided are host vector systems useful for the recombinant production of the recombinant polypeptides in procaryotic and eucaryotic systems. Cells comprising the host vector systems of this invention as well as methods of recombinantly producing these polypeptides are provided by this invention. Further provided is a method to detect the recombinant polypeptides of this invention. Further provided is a method of screening for a compound which inhibits or augments the formation of β-amyloid protein.

BRIEF DESCRIPTION OF THE FIGURES

[0007] Figure 1: Schematic representation of APP-REP 751. APP-REP 751 represents a cleavable APP substrate system which contains target sequences of BAP including normal flanking regions (not to scale). The APP-REP protein is marked with a 276 amino acid deletion (corresponding to APP-751 beginning at XhoI through to and including the glycine codon at 15 amino acid residues N-terminal to BAP) and the insertion of sequences encoding N- and C- terminal reporter epitopes. Substrate P (SP) reporter epitope (RPKPQQFFGLM) is inserted at the XhoI site. Met-enkephaline (ME) reporter epitope (YGGFM) is inserted at the C-terminus of APP. The resulting construct encodes 492 amino acids (see Figure 2).

[0008] Figure 2: Schematic representation depicting the construction of APP-REP from APP-751 cDNA. Partial representing N- and C-terminal regions of APP-REP were cloned separately as illustrated below. The N-terminal partial was constructed by ligating sequences encoding substance P (SP) to an N-terminal fragment of APP cDNA. The C-terminal partial was constructed by PCR amplification using the corresponding portion of APP cDNA to introduce novel ends including the Met-enkephalin (ME) reporter epitope. A functional APP-REP 751 clone was obtained by subcloning the partials as indicated. EcoRI (E), XhoI (X), HindIII (H), BamHI (B), SalI (S), XbaI (Xb).

[0009] Figure 3: Epitope mapping of APP-REP 751 expressed in COS-1 cells. Immunoprecipitation analysis of cell lysate and conditioned medium using the SP (anti-N-terminal substance P reporter) and M3 (anti-C-terminal APP) antisera. Lanes 1 and 2, cell lysate immunoprecipitated with SP and M3 antisera, respectively; lanes 3 and 4, conditioned medium immunoprecipitated with M3 and SP antisera, respectively; lanes 5 and 6, conditioned medium of control cells transfected with vector DNA immunoprecipitated with SP and M3 antisera, respectively; lane M, molecular weight markers.

[0010] Figure 4: Pulse-chase analysis of APP-REP 751. Immunoprecipitation of cell lysate (A) and CM (B). COS-1 cells were pulsed with [^{35}S]-methionine for 15 minutes and chased using cold methionine for 0, 0.5, 1, 1.5, 2 and 4 hours (lanes 1 to 6). Lanes 7, 8 and 9 are chase intervals of 0, 1 and 2 hour for control cells transfected with vector DNA. Lane M, molecular weight markers.

[0011] Figure 5: Epitope mapping and comparative expression of APP-REP 751, $BAP_{E22Q}$and BAP_{Δ}^{11-28}.A, Schematic representation of relevant BAP (boxed) and flanking amino acid sequences of APP-REP 751, $BAP_{E22Q}$and $BAP_{\Delta 11-28}$juxtaposed against the putative transmembrane domain (shadowed). B-F, Immunoprecipitation analysis with antibodies recognizing indicated substance P (SP), KPI domain (KPI), C-terminal APP (M3) or Met-enkephalin (ME) epitopes; Lane M, molecular weight marker. B, Conditioned medium obtained from COS-1 cells expressing APP-REP 751 (lane 3), BAP_{E22Q}(lanes 4, 6 and 8), $BAP_{\Delta 11-28}$(lanes 5, 7 and 9) or control cells with (lane 2) or without (lane 1) transfection with vector DNA. C, Cell lysates obtained from COS-1 cells expressing APP-REP BAP_{E22Q}(lanes 1, 4 and 7), BAP_{Δ} 11-22 (lanes 2, 5 and 8) and control cells transfected with vector DNA (lanes 3, 6 and 9). D, Accumulation of secreted APP-REP 751 fragments in the conditioned medium obtained from COS-1 cells expressing APP-REP 751 (lanes 2 and 6), BAP_{E22Q} (lanes 3 and 8), $BAP_{\Delta 11-28}$(lanes 4 and 7), or control cells transfected with vector DNA (lanes 1 and 5), were pulsed with [^{35}S]-methionine and chased for 45 (lanes 1-4) or 90 (lanes 5-8) minutes with cold methionine. E, Accumulation of secreted APP-REP fragments in the conditioned medium obtained from stable (Chinese hamster ovary cells; lanes 1-4) and transient (COS-1 cells; lanes 5 and 6) expression of APP-REP 751 (lanes 2 and 5), $BAP_{\Delta 11-28}$ (lanes 3 and 6), BAP_{E22Q} (lane 4), or control cells transfected with vector DNA (lane 1).

[0012] Figure 6: Peptide mapping and sequencing of fragments secreted into the conditioned medium obtained from Chinese hamster ovary cells stably expressing APP-REP 751, BAP_{E22Q} and $BAP_{\Delta 11-28}$. A, Schematic representation depicting the APP-REP 751 and related derivative indicating the cleavage products and relevant carboxy-terminal fragments derived from treating the secreted fragments either with BNPS-Skatole (B) or cyanogen bromide. Downward- or upward-facing arrows represent BNPS-Skatole and cyanogen bromide cleavage sites, respectively. Amino acid lengths of relevant fragments for mapping or sequencing are given. B, BNPS-Skatole treatment of fragments secreted into the conditioned medium obtained from CHO cells stably expressing APP-REP 751 or $BAP_{\Delta 11-28}$. Mixture of conditioned medium containing APP-REP and $BAP_{\Delta 11-28}$(lane 1), or $BAP_{\Delta 11-28}$(lane 2) and APP-REP 751 (lane 3) alone.

[0013] Figure 7: Nucleotide and amino acid sequence of the APP-REP 751 protein.

[0014] Figure 8: Nucleotide sequence of APP 770 which also is available from the Genebank data base under accession number Y00264.

DETAILED DESCRIPTION OF THE INVENTION

[0015] This invention provides a nucleic acid molecule encoding an amyloid precursor mutein, wherein the nucleic acid molecule comprises, from the 5' end to the 3' end, a nucleic acid sequence encoding a marker and a nucleic acid sequence encoding the amino terminus of APP up to but not including the sequences that encode BAP. These nucleic acid molecules may include, but are not limited to the nucleic acid molecules selected from the group consisting of pCLL983, pCLL935, pCLL934 and pCLL913.

[0016] This invention also provides a nucleic acid molecule encoding an amyloid precursor mutein, wherein the nucleic acid molecule comprises, from the 5' end to the 3' end a nucleic acid sequence encoding BAP and a nucleic acid sequence encoding a marker. These nucleic acid molecules may include, but are not limited to the nucleic acid molecules selected from the group consisting of pCLL947, pCLL914, pCLL937, pCLL949 and pCLL957.

[0017] Further provided by this invention is a nucleic acid molecule which comprises the nucleic acid molecules defined hereinabove to each other. Method of ligating are well known to those of skill in the art. These nucleic acid molecules may include, but are not limited to the nucleic acid molecules selected from the group consisting of pCLL618, pCLL619, pCLL620, pCLL600, pCLL964, pCLL962, pCLL989, pCLL987, pCLL990, pCLL988, pCLL601, pCLL602, pCLL603, pCLL604, pCLL605, pCLL606 and pCLL607. These nucleic acid molecules are described in Table 3.

[0018] As used herein, the term "amyloid precursor mutein" is intended to encompass an amyloid precursor protein that is mutated, i.e., it is derived from a nucleic acid molecule which has changes in its primary structure as compared to wild-type amyloid precursor protein (APP). Wild-type APP exists in three isoforms, thus, the nucleic acid molecule is changed in its primary structure for each of the three isoforms of wild-type APP. As is known to those of skill in the art, a mutation may be a substitution, deletion, or insertion of at least one nucleotide along the primary structure of the molecule. The mutations which are encompassed by this invention are the result of saturation mutagenesis in the regions of APP which are susceptible to cleavage by endoproteolytic enzymes. These mutations include deletions of nucleic acids encoding particular amino acids, substitution of nucleic acid sequences encoding one amino acid for a different amino acid and addition of nucleic acid sequences encoding additional amino acids not present in the wild type APP sequence. The term "marker" encompasses any substance capable of being detected or allowing the nucleic acid or polypeptide of this invention to be detected. Examples of markers are detectable proteins, such as enzymes or enzyme substrates and epitopes not naturally occurring in wild-type APP that are capable of forming a complex with an antibody, e.g. a polyclonal or monoclonal antibody. In the preferred embodiment of this invention, the marker is an epitope that is capable of being detected by a commercially available antibody. In one embodiment, the marker is an epitope capable of being detected by a monoclonal antibody directed to the Substance P, the Met-enkephalin or the c-myc epitope. In the most preferred embodiment of this invention, the marker is the c-myc epitopic region.

[0019] The term "BAP region" is defined as the region of APP wherein endoproteolytic cleavage will yield the amino-terminus and the carboxy-terminus of the BAP which is deposited as plaques and cerebrovascular amyloid in Alzheimer's disease brain. The function of the "BAP region" is to give rise to BAP which may function as a neurotoxic and/ or neurotrophic agent in the brain and as other functionalities ascribed to BAP. The "BAP region" may also be endoproteolytically cleaved by enzymes. Such enzymes may include, but are not limited to the enzymes multicatalytic prtenase, propyl-endopeptidase, Cathepsin-B, Cathepsin-D, Cathepsin-L, Cathepsin-G or secretase. Secretase cleaves between lysine-16 (K-16) and leucine-17 (L-17) where full length BAP comprises the amino acid sequence DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA. Thus, for the purposes of this invention, the preferred embodiment is a cDNA which encodes an RNA which is translated into a protein which is the substrate for endoproteolytic activities which generate BAP.

[0020] In addition, for the purposes of this invention, the nucleic acid molecule may be DNA, cDNA or RNA. However, in the most preferred embodiment of this invention, the nucleic acid is a cDNA molecule.

[0021] This invention also encompasses each of the nucleic acid molecules described hereinabove inserted into a vector so that the nucleic acid molecule may be expressed, i.e., transcribed (when the molecule is DNA) and translated into a polypeptide in both procaryotic and eucaryotic expression systems. Suitable expression vectors useful for the practice of this invention include pSVL (Pharmacia), pRCRSV (Invitrogen), pBluescript SK$^+$ (Stratagene), pSL301 (Invitrogen), pUC19 (New England Biolabs). However, in the preferred embodiment of this invention, the vector pcDNA-1-neo is the expression vector for expression in eucaryotic cells. As is well known to those of skill in the art, the nucleic acid molecules of this invention may be operatively linked to a promoter of RNA transcription, as well as other regulatory sequences. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct the transcription of RNA off of the nucleic acid molecule. An example of a promoter is the human cytomegalovirus promoter. The vectors of this invention preferably are capable of transcribing and/or translating nucleic acid in vitro or in vivo. The recombinant polypeptides produced from the expression of the nucleic acid molecules of this invention are also provided.

[0022] A host vector system for the production of the recombinant polypeptides described hereinabove and for expressing the nucleic acid molecules of the subject invention are provided. The host vector system comprises one of the vectors described hereinabove in a suitable host. For the purpose of the invention, a suitable host may include, but is not limited to a eucaryotic cell, e.g., a mammalian cell, a yeast cell or an insect cell for baculovirus expression. Suitable mammalian cells may comprise, but are not limited to Chinese hamster ovary cells (CHO cells), African green monkey kidney COS-1 cells, and ATCC HTB14 (American Type Tissue Culture). Most preferably, the cell lines CRL 1650 and CRL 1793 are used. Each of these are available from the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, Maryland U.S.A. 20852. Suitable procaryotic cell may include, but are not limited to bacteria cells, HB101 (Invitrogen), MC1061/P3 (Invitrogen), CJ236 (Invitrogen) and JM109 (Invitrogen). Accordingly, the procaryotic or eucaryotic cell comprising the vector system of this invention is further provided by this invention.

[0023] As is known to those of skill in the art, recombinant DNA technology involves insertion of specific DNA sequences into a DNA vehicle (vector) to form a recombinant DNA molecule which is capable of being replicated in a host cell. Generally, but not necessarily, the inserted DNA sequence is foreign to the recipient DNA vehicle, i.e., the inserted DNA sequence and DNA vector are derived from organisms which do not exchange genetic information in nature, or the inserted DNA sequence comprises information which may be wholly or partially artificial. Several general methods have been developed which enable construction of recombinant DNA molecules. For example, U.S. Patent No. 4,237,224 to Cohen and Boyer describes production of such recombinant plasmids using processes of cleavage of DNA with restriction enzymes and joining the DNA pieces by known method of ligation.

[0024] These recombinant plasmids are then introduced by means of transformation or transfection and replicated in unicellular cultures including procaryotic organisms and eucaryotic organisms and eucaryotic cells grown in tissue culture. Because of the general applicability of the techniques described therein, U.S. Patent No. 4,237,224 is hereby incorporated by reference into the present specification. Another method for introducing recombinant DNA molecules into unicellular organisms is described by Collins and Hohn in U.S. Patent No. 4,304,863 which is also incorporated herein by reference. This method utilized a packaging, transduction system with bacteriophage vectors (cosmids).

[0025] Nucleic acid sequences may also be inserted into viruses, for example, a vaccinia virus or a baculovirus. Such recombinant viruses may be generate, for example, by transfection of plasmids into cells infected with virus, Chakrabarti et al, (1985) Mol. Cell Biol. 5:3402-3409.

[0026] Regardless of the method used for construction, the recombinant DNA molecule is preferably compatible with the host cell, i.e., capable of being replicated in the host cell either as part of the host chromosomes or as an extra-chromosomal element. The recombinant DNA molecule or recombinant virus preferably has a marker function which allows the selection of the desired recombinant DNA molecule(s) or virus, e.g., baculovirus. In addition, if all of the proper replication, transcription and translation signals are correctly arranged on the recombinant DNA molecule, the foreign gene will be properly expressed in the transformed or transfected host cells.

[0027] Different genetic signals and processing events control gene expression at different levels. For instance, DNA transcription is one level, and messenger RNA (mRNA) translation is another. Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes RNA synthesis. The DNA sequences of eucaryotic promoters differ from those of procaryotic promoters. Furthermore, eucaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a procaryotic system.

[0028] Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno (SD) sequence on the mRNA. For a review on maximizing gene expression, see Roberts and Lauer (1979) Methods in Enzymology 68:473.

[0029] Many other factors complicate the expression of foreign genes in procaryotes even after the proper signals are inserted and appropriately positioned. One such factor is the presence of an active proteolytic system in E. coli and other bacteria. This protein-degrading system appears to destroy foreign proteins selectively. A tremendous utility, therefore, would be afforded by the development of a means to protect eucaryotic proteins expressed in bacteria from proteolytic degradation. One strategy is to construct hybrid genes in which the foreign sequence is ligated in phase (i.e., in the correct reading frame) with a procaryotic structural gene.

[0030] Expression of this hybrid gene results in a recombinant protein product (a protein that is a hybrid of procaryotic and foreign amino acid sequences).

[0031] Successful expression of a cloned gene requires efficient transcription of DNA, translation of the mRNA and in some instances post-translation modification of the protein. Expression vectors have been developed to increase protein production from the cloned gene. In expression vectors, the cloned gene is often placed next to a strong promoter which is controllable so that transcription can be turned on when necessary. Cells can be grown to a high density and then the promoter can be induced to increase the number of transcripts. These, if efficiently translated, will result in high yields of polypeptide. This is an especially valuable system if the foreign protein is deleterious to the host cell.

[0032] Several recombinant DNA expression systems are described below in the Experimental Procedures section for the purpose of illustration only, and these examples should not be construed to limit the scope of the present invention.

[0033] A method for producing a recombinant polypeptide described hereinabove, is also provided. This method comprises growing the host cell containing the nucleic acid of this invention and/or the host vector system of this invention under suitable conditions, permitting production of the polypeptide and recovering the resulting recombinant polypeptide produced.

[0034] A method of detecting in a sample the presence of any of the recombinant polypeptides described hereinabove is further provided by this invention. In the preferred embodiment of this invention, the marker is an epitope directed against an antibody, the epitope of which is not present in the wild-type polypeptide or APP derivative. This method comprises obtaining a sample suspected of containing the polypeptide and contacting the sample with an antibody directed to the marker. The contacting is done under suitable conditions to favor the formation of an antibody-epitope (i.e., antigen) complex, and detecting the presence of any complex so formed. The presence of complex being a positive indication that the recombinant polypeptide is in the sample. In one embodiment of this invention, the antibody is a mouse antibody. In another embodiment of this invention, the antibody is a human antibody. In the most preferred embodiment, the mouse or human antibody is either a mouse or human monoclonal antibody.

[0035] The antibody is labeled with a detectable marker selected from the group consisting of radioisotopes, dyes, enzymes and biotin. For the purposes of this invention, suitable radioisotopes include, but are not limited to, ^{32}P, ^{35}S, ^{131}I and ^{125}I.

[0036] Suitable samples for the practice of this invention include, but are not limited to conditioned media, cell lysates and cellular organelle fractions.

[0037] The method of this invention may utilize the recombinant polypeptide for the detection of drugs or compounds that inhibit or augment the activity of proteolytic enzymes which cleave APP to generate BAP fragments. For the purposes of example only, a recombinant polypeptide which contains a Substance-P marker epitope on the amino-terminal side of BAP and a Met-enkephalin marker epitope on the carboxy-terminal side of BAP. Using commercially available RIA kits (Peninsula), one can measure the amount of amino-marker and carboxy-marker in any given sample. Since endoproteolytic activity is shown (see Figure 3) to allow the release of amino-terminal fragments of APP containing the amino-marker into the conditioned media while carboxy-terminal APP fragments containing the carboxy-marker remain associated with the cell, then RIA measures the amount of amino-marker in the conditioned medium as a direct result of endoproteolytic cleavage activity between the marker epitopes preferably within the "BAP region". Using this RIA to the amino-marker, the effect of potential drugs designed to modify endoprotease activity can be tested comparing the level of amino-marker in untreated and endoprotease-inhibitor treated samples. If a difference in non-treated and treated samples is found, then the position of the cleavage or lack of cleavage can be verified as with the procedures used in Figures 3 to 6. Thus, the qualitative and quantitative aspects of endoproteolytic activity and its inhibition on the recombinant APP mutein is evaluated. The amino-marker also is an enzyme such as beta-galactosidase which would be released in the conditioned media by the action of an appropriate endoprotease. Cell free samples of conditioned media containing the liberated enzyme converts a chromogenic substrate into the appropriately colored product (Blue for X-gal and Yellow for ONPG) which is measured spectrophotometrically. Inhibitors of the appropriate endoprotease would inhibit the release of beta-galactosidase enzyme into the conditioned medium resulting in less colored product being observed.

[0038] It is a purpose of this invention to develop a cleavable APP substrate system which represents target sequences of BAP including normal flanking regions to provide recognition sequences for processing enzymes. The utilization of a common substrate for parallel strategies involving in vitro cleavage assays using cellular extracts, in vivo processing assays in tissue culture or bacterial cells, or in conjunction with a selection system aimed at cloning BAP-cleaving proteases (or other relevant proteins) is preferred.

[0039] A second purpose of this invention is to develop an APP substrate which is non-cleavable by secretase in order to better detect other putative abnormal processing events which are hypothesized to potentially either compete with secretase for limited substrate, or occur at much lower frequency than secretase and whose effects may be otherwise masked by the mass action of secretase. These are referred to as "secretase-minus mutants" in Table 4.

[0040] Third, secretase-cleavable and -noncleavable APP substrates would provide probes with which to investigate cellular post-translational modifications to APP in an attempt to determine the potential influence on normal secretase and abnormal BAP "clipping" activities. These areas include, among others, the consideration of various known APP point mutations, contribution by different cell/tissue types (normal- or AD-specific), the Kunitz Protease Inhibitor domain present in APP-770 and - 751 isoforms, APP phosphorylation and APP glycosylation.

[0041] These are referred to as "APP 717 mutations" or Dutch Disease Mutations in Table 5.

[0042] Fourth, the ability to detect specific APP proteolytic events, either the normal secretase or the abnormal BAP-generating activities, would enable the use of strategies which use phenotypic rescue as a marker for the cloning of potentially relevant and interesting proteases in tissue culture systems.

Overview of the APP-REP Strategy

[0043] To study secretase and BAP-generating pathways, portions of APP cDNA clones are used to engineer a panel of APP-REPorter (APP-REP) plasmids to express "marked" proteins representing each of the APP isoforms (and other APP/BAP sequence alterations; see below) in cultured cells. The system utilizes the marker Substance-P (SP) and Met-Enkephalin (ME) which are strategically placed, respectively, on amino- and carboxy-terminal sides of BAP. Proteolytic cleavage of APP-REP target substrate is determined by the electrophoretic sizing of resulting proteolytic fragments and immunological detection of APP-specific and SP and ME reporter epitopes. Deletion of a large central portion of APP sequence also makes APP-REP readily distinguishable from the endogenous APP isoforms based on size. Moreover, the resolution of detecting proteolytic cleavage at different positions within the APP-REP substrate protein is enhanced by working with shorter target substrates. Approximate location of cleavage is determined initially by fragment sizing and epitope mapping; the exact cleavage site is later determined by peptide mapping of affinity/HPLC purified fragments and sequencing of peptide ends.

[0044] Plasmids also are derived from these constructs for developing similar strategies to express APP-REP protein in cell free reticulocyte transcription-translation and bacterial systems. Mutation of APP-REP secretase/BAPase cleavage site (by sequence substitution, deletion or FAD mutations) can reveal putative proteolytic activities associated with BAP formation including amino- and carboxy-BAPase activities which are predicted to result in altered product fragments lengths.

FIRST SERIES OF EXPERIMENTS

Bacterial Strains and Transformation

[0045] Transformation of commercially available frozen competent bacteria, maintenance and selection of transformants is according to the manufacturer. Strains HB101, DH5a or JM109 (Gibco-BRL) are used for the construction of APP-REP in pSK(+) (Stratagene, La Jolla, CA) and pSL 301 (Invitrogen, San Diego, CA). APP-REP is subsequently subcloned into the eucaryotic expression vector pcDNA-1-neo and amplified in MC1061/P3 (Invitrogen, San Diego, CA).

Plasmid Construction

[0046] A cassette approach is used to independently construct portions of the APP-REP plasmid (Figure 2). The N-terminal partial includes APP sequences through the Substance P (SP) epitope, while the carboxy-terminal (C-terminal) partial includes BAP (or sequence variations of BAP) through the Met-enkephalin (ME) epitope (Figure 1). A plasmid encoding the N-terminal cassette (either of the plasmids listed in Table 1A, which include pCLL983, pCLL935, pCLL934 and pCLL913) is constructed by ligating the EcoRI-XhoI fragment derived from each of the corresponding APP cDNAs listed in Table 1A,which include APP-695, APP-751 or two different APP-770 sequences,to a short synthetic XhoI-HindIII fragment encoding Substance P (amino acid 1-11). This product is then ligated into the EcoRI and HindIII sites of pSK(+). Plasmid encoding the carboxy-terminal (C-terminal) cassette (pCLL947) is constructed by cloning into the HindIII-BamHI sites of pSL301 a fragment containing BAP sequences which is amplified by polymerase chain reaction.

[0047] The fragments feature a novel 5'-HindIII site beginning at lysine 638 of APP-751, native or modified BAP sequences, and additional full-length or truncated APP sequences. The C-terminal cassette provides APP C-terminal wild type sequences, truncations following the transmembrane domain of BAP sequence, an E to Q substitute at BAP as#22, or a G to A substitute at BAP aa#10 with the deletion of aa#11-28 and creation of a novel NdeI site. Each of the APP C-terminal variantes contain the additional Met-enkephalin sequences. Each of the resulting pSL301 HindIII-SalI fragments (including HindIII-BamHI coding region plus BAmHI-SalI polylinker sequences) is then isolated and ligated pairwise with each of the N-terminal cassettes by subcloning into the HindIII-SalI sites of the SK(+)-based plasmid to generate the panel of new plasmids identified in Table 2. Next, the polylinker of the CMV promotor driven eukaryotic expression vector, pcDNA-1-neo (pCLL601), was modified to accommodate the panel of plasmids listed in Table 2 of XbaI-SalI APP and APP-Rep fragments to create a second panel of plasmids listed in Table 3 for eukaryotic expression.

[0048] Polylinker modification involves the substitution of the HindIII-XbaI fragment with a synthetic one which restores HindIII, destroys XbaI and introduces novel BamHI-XabI-Xho-SalI sites.

Tissue Culture Lines

[0049] All cells are obtained from American Type Culture Collection and maintained according to their recommendation. They include SV40-transformed African Green monkey kidney COS-1 cells (CRL 1650) for transient expression and Chinese hamster ovary CHO-1C6 (CRL 1973) for stable expression systems.

Transfection Procedure

[0050] Cells are seeded at a density of 2-3 X 10^6/100 mm dish and transfected using Lipofectin (Gibco-BRL, Grand Island, NY) when ~75% confluent. Plasmid DNA (0.5-4 mg) is diluted in 450 ml of Opti-MEM (Gibco-BRL, Grand Island, NY), mixed with 450 ml containing 75-100 ml Lipofectin and the mixture incubated at room temperature for 20-30 minutes. Addition of DNA-Lipofectin mixture to cells, recovery phase and G418 selection (Gibco-BRL), when applicable, are according to the manufacturer's protocol. Cells and conditioned medium are harvested at 48-72 hours following transfection for assay of APP-REP expression.

Antisera

[0051] APP-specific antisera:anti-N-terminal APP, mouse monoclonal 22C11 (Boehringer-Mannheim Biochemicals, Indianapolis, IN) raised against a recombinant fusion protein expressing APP-695 (epitope mapped to aa 60-100); anti-KPI rabbit polyclonal, raised against recombinant protein encoded by the HinfI fragment derived from APP-770; and anti-APP C-terminal rabbit polyclonal M3, raised against synthetic APP peptides corresponding to APP-770 amino acid residues 649-671. Reporter-specific antisera:anti-substance P, rabbit polyclonal, purchased from Peninsula, Belmont, CA; and anti-Met-enkephalin, rabbit polyclonal, purchased from Cambridge, Wilmington, DE.

[0052] Preparation of Radiolabeled APP-REP and Extraction from Conditioned Medium and Cell Lysates

[0053] APP-REP proteins transiently expressed in exponentially growing adherent cells (~4 x 10^6) are radiolabeled by metabolic incorporation of [^{35}S]-methionine as follows. Cell monolayers are washed twice with prelabeling medium (methionine-free D-MEM supplemented with glutamine, sodium pyruvate, antibiotics and 1% dialyzed fetal bovine serum (Gibco-BRL) and incubated for 15 minutes to 4 hours in prelabeling medium containing 150-450 uci[^{35}S]-methionine (Amersham, 800Ci/mmol). If chased with cold methionine, the medium is removed following the pulse, the monolayer is washed with prelabeling medium and replaced with 3 ml of the same containing 1 mM cold methionine.

[0054] The conditioned medium is recovered following radiolabeling by aspiration from plates and cell debris removed by centrifugation for 10 minutes at 4°C (-300xg). Conditioned medium is immediately supplemented with protease inhibitors (pepstatin A, 50 ug/ml; leupeptin, 50 ug/ml; aprotinin, 10 ug/ml; EDTA, 5 mM; PMSF, 0.25 mM) and immunoprecipitation buffer (IPB; Sisodia et al., 1990) for protein analysis. Briefly, 3 ml of CM is supplemented with 0.75 ml 5X IPB (250 mM Tris, pH 6.8; 750 mM NaCl; 25 mM EDTA; 2.5% Nonidet P40; 2.5% sodium deoxycholate) and incubated for 20 minutes at 4°C prior to use.

[0055] Lysates are prepared by washing the labeled cell monolayer twice with 5 ml pre-labeling medium and directly extracting cells in plates at 4° C with 3.75 ml 1X IPB (including protease inhibitors). Cells are scraped into the buffer, incubated for 20 minutes at 4°C and lysates clarified of cellular debris by centrifugation for 20 minutes at 10,000xg.

[0056] For radioiodination of cell surface proteins, monolayers are chilled on ice, washed 3 times with 5 ml ice cold PBS and labeled at room temperature for 10 minutes following the addition of: 5 ml PBS containing 0.2 mCi Iodine-125 (NEZ-033A, New England Nuclear), 0.25 ml lactoperoxidase (1 mg/ml distilled water, Sigma), 10 ul of hydrogen peroxide solution (freshly prepared by diluting 10 ml of 30% stock in 10 ml of PBS) added at 0, 3, 6, and 9 minutes of iodination. At 10 minutes, the supernatant is removed and cells gently washed with 10 ml of ice cold PBS (containing 10 mM NaI). Four ml of PBS is added, and CM and cell lysates are prepared as above.

Immunoprecipitation Analysis

[0057] Aliquots of radiolabeled lysate or conditioned medium representing 4-8x10^5 cells are thawed on ice, supplemented with protease inhibitors (see above), boiled for 3 minutes in 0.35% SDS and chilled on ice. Samples are preincubated on a shaker for 1.5 hours at 4°C with 2-10 ul 2X of preimmune (or normal rabbit) serum and 2 mg Protein A-Sepharose (Sigma; prepared in 1X IPB), and insoluble immune removed by centrifugation. APP-or reporter epitope-specific antisera (0.1-10 ul) and 2 mg Protein A-Sepharose were similarly added and incubated overnight. Specific immune complexes were precipitated, washed 4 times with 0.25 ml 1 X IPB (with protease inhibitors), extracted with 20 ul Laemmli sample buffer (Laemmli (1970) Nature 227:680-685), boiled for 3 minutes and fractionated by electrophoresis on SDS-polyacrylamide-tris-glycine (Bio-Rad Laboratories, Richmond, VA) or SDS-polyacrylamide-tris-tricine Daiichi (Integrated Separation Systems, Natick, MA) gels. Gels are then treated with Enlightening Autoradiographic Enhancer (New England Nuclear, NEF-974) and dried in vacuo with heat and exposed to Kodak X-AR film at -70°C.

Western (Immunoblot) Analysis

[0058] Lysate or 10X concentrated conditioned medium (Centricon 30 microconcentrator; Amicon, Beverly, MA) representing 4-8x10^5 cells are supplemented with an equal volume of 2X Laemmli sample buffer, boiled for 2 minutes, fractionated by electrophoresis on SDS-polyacrylamide-tris-glycine (Bio-Rad, XX) or SDS-polyacrylamide-tris-tricine Daiichi (Integrated Separation Systems, Natick, MA) gels and transblotted (Semi-Phor, Hoefer Instruments, San Francisco, CA) to Immobilon-P membrane (Millipore, Bedford, MA). Membranes are pre-blocked in 10 ml 5% non-fat dry milk/PBST (PBS with 0.02% Tween-20) for 45 minutes at room temperature prior to overnight incubation at 4°C with primary antisera (in fresh pre-blocking solution). Blots are then washed, incubated with secondary antibody, washed and developed for horseradish peroxidase activity as described (ECL Luminol Kit; Amersham, Arlington Heights, IL).

Peptide Mapping and Determination of the Site of Proteolytic Cleavage by Peptide Sequencing

[0059] The secretase clip site is determined essentially as described (Wang et al., (1991) J. Biol. Chem. 266: 16960-16964). Approximately 1X10^6 CHO cells stably expressing APP-REP are seeded in each 150 mm dish containing DMEM (complete with 200 ug/ml G418) and incubated for 36 hours. Cells are washed, preincubated for 6 hours in serum-free medium (MCDB 302 supplemented with antibiotics, L-glutamine (292 mg/l) and proline 12 mg/l (Sigma) to remove serum components, washed, and incubated for another 72 hours in fresh serum-free media.

[0060] Serum-free conditioned medium was pooled and cell debris was removed by centrifugation (10 minutes at 300xg, then 30 minutes at 100,000xg) and concentrated by acetone precipitation and fractionated by FPLC. Conditioned medium concentrate is loaded on an anion exchange column (Mono Q; source) and protein is eluted in 20 mM Tris (pH 7.4) over a 0-1M NaCl gradient. Fractions containing secreted APP are identified by immunoblotting (monoclonal antibody 22C11) and relevant samples pooled, desalted (NP-5 column; Pharmacia, Piscataway, NJ) and concentrated. Proteins are then denatured, treated with cyanogen bromide (in 10% trifluoroacetic acid) and peptides separated by high performance liquid chromatography (Vydac C_{18} reverse-phase) attached to a FAB-MS unit. Relevant peaks derived from APP-REP 751 and APP-REP BAP $_{11\text{-}28}$are identified by locating these peaks uncommon to both proteins. The C-terminal peptides derived from APP-REP BAP $_{11\text{-}28}$(predicted 14 amino acid) and APP-REP 751 (predicted 17 amino acid) are sequenced (MilliGen solid phase peptide sequencer; Millipore, Burlington, MA).

EXPERIMENTAL RESULTS

Characterization of APP-REP Expression by Epitope Mapping

[0061] The APP-REP strategy (Figure 1) is a system for the expression of marked APP proteins in tissue culture cells in order to characterize the proteolytic cleavage events. The deletion of a 276 amino acid portion distinguishes the construct of this invention from endogenously expressed APP on the basis of size, and is predicted to increase the resolution of APP-REP fragments resulting from the proteolytic cleavage by secretase or other amyloidogenic, BAP-generating cleavage events. Substance P and Met-enkephalin marker epitopes strategically placed on either side of BAP enable the immunological detection of N- and C-terminal fragments, respectively, which result from proteolytic cleavage of APP-REP substrate.

[0062] APP-REP protein transiently expressed in COS-1 cells has been radiolabeled by metabolic incorporation of [^{35}S]-methionine in a 60 minute pulse, immunoprecipitated with antisera, and size fractionated by gel electrophoresis as demonstrated in Figure 3. Immunoprecipitation with a panel of APP- and APP-REP-specific antisera which recognize epitopes mapping at various positions along APP-REP, reveals the presence of 2 proteins of ~63 kDa in cell lysates (including cytoplasmic and membrane associated proteins) as shown in Figure 3. The specific detection by antisera directed against the KPI domain, the carboxy-terminus of APP (M3, Figure 3A) and Met-enkephalin, as well as by the N-terminal 22C11 monoclonal in Western blot analysis (data not shown), suggests that both bands represent the full-length APP-REP protein. Although the 492 amino acid APP-REP is predicted to display a mobility of -49-54 KdA, the larger 63 and 76 kDa proteins are expected based on previous observations attributing the aberrant migration properties of APP, putatively to post-translational modification like tyrosine-sulfation, glycosylation and phosphorylation (Dyrks et al., (1988) EMBO J. 7:949-957; Weidemann et al., (1989) Cell 57:115-126.

[0063] Analysis of the conditioned medium (CM) collected from those same cells above indicates that an N-terminal fragment of APP-REP is released into the CM. Figure 3B reveals a shorter -67 kDa fragment immunoprecipitable from CM with KPI and SP antisera (and the 22C11 monoclonal by Western analysis), but not with several C-terminal APP or ME antisera. These data are consistent with the observations (Selkoe et al., (1988) P.N.A.S. 86:6338-6342; Palmert et al., (1989 a) P.N.A.S. U.S.A. 85:7341-7345), b) indicating that APP is a substrate for the proteolytic cleavage resulting in the secretion of an N-terminal fragment into CM, and a short membrane associated C-terminal fragment.

Pulse-Chase Analysis Reveals the Precursor/Product Relationship Between Cell Associated and Secreted Derivatives of APP-REP

[0064] To show that APP-REP undergoes post-translational modification accounting for the 2 cell associated proteins, and that the N-terminal APP-REP fragment released into CM is derived from one of these precursors, radiolabeled APP-REP is with a short 15 minute pulse and collected both cell lysates and CM at various chase intervals as shown in Figure 4. Immunoprecipitation analysis reveals that APP-REP initially migrates at -63 kDa and is rapidly "chased" up to ~76 kDa with conversion rate of less than 10-15 minutes (Figure 4A; also see Figure 5C for quantitative analysis), an observation which is consistent with the notion that APP-REP, like APP, is a substrate for post-translational modifications.

[0065] The -76 kDa APP-REP band (cell lysate) rapidly disappears (t $^{1/2}$ -20 minutes) (Figure 4A and 5C), followed by the appearance of a shorter -67 kDa band in the CM (Figure 4B and 5C). The released -67 kDa fragment accumulates rapidly and is relatively long lived (t 1/2 > 8 hours). The temporal pattern of intracellular APP-REP depletion, accumulation of a shorter ~67 kDa protein in CM, and the recognition of this protein only by antisera raised against N-terminal epitopes, is consistent with proteolytic cleavage of APP-REP which is similar to the normal, non-amyloidogenic, "secretase" activity which results in the release of an N-terminal APP fragment (Sisodia et al., (1990) Science 248:492-495.

Expression of APP-REP Derivatives Containing Altered BAP Sequences Does Not Prevent Proteolytic Cleavage

[0066] In an attempt to engineer non-cleavable substrates for secretase, APP-REP proteins are expressed (Figure 5A) either lacking the secretase "cleavage/recognition site" putatively encompassed by aa residues BAP 11-28 ($BAP_{\Delta 11\text{-}28}$pCLL604), or representing the BAP point mutation found in patients with HCHWA-D (BAP $_{E22Q}$:pCLL603).

The construct representing the BAPE22Q mutation results in secretion of an N-terminal fragment indistinguishable from the APP-REP protein (Figure 5C). Deletion of extracellular, juxtamembranous 18 aa (BAP_{Δ} 11-28), however, still results in the secretion of an N-terminal APP-REP fragment into the CM (Figure 5B). A slightly faster migration of fragment derived from the deletion construct pCLL604 in comparison to that of wild-type pCLL602, is consistent with the 18 aa deletion and a corresponding loss of ~2 kDa (Figure 5C). Pulse-chase analyses (Figure 5D) indicate that expression of full-length precursor by each construct, proteolytic cleavable and the release of fragment into CM is both qualitatively and quantitatively similar to that of the wild-type APP-REP sequence. Chinese hamster ovary (CHO) cells stably expressing APP-REP display results similar to that of transiently expressing COS-1 cells (Figure 5E). Collectively, these data suggest that the cleavage in each case may be the result of similar biochemical events despite the difference in juxtamembranous sequences (Figure 5A).

Full-Length APP-REP Proteins Are Associated with Plasma Membrane Prior to Cleavage

[0067] In preliminary experiments, detection of the amino-terminal APP-REP fragment in CM and not in cell lysates, suggests that the putative secretase activity might be plasma membrane-associated. One prediction of this notion is that an N-terminal portion of APP-REP might be (partially) localized to the extracellular environment prior to cleavage. In order to test this hypothesis, CHO cells stably expressing APP-REP (pCLL602) are subjected to lactoperoxidase-catalyzed iodination to radiolabel only extracellular proteins associated with the cell surface, and CM and cell lysates were analyzed immediately following iodination or after a 10 minute incubation. Presence of the ~76 kDa APP-REP band in cell lysate should indicate that at least a portion of full-length APP-REP is poised extracellularly in association with cell membrane. Detection of both, a reduced fraction of the ~76 kDa band in the cell lysate and a corresponding increased fraction of ~67 kDa fragment in CM following the "release" incubation, would suggest that the extracellular portion of APP-REP is cleaved.

Peptide Sequencing to Determine the Site of Proteolysis

[0068] Fragment secreted into serum-free media derived from CHO cells stably expressing APP-REP with wild-type or BAP 11-28 sequences has been analyzed to determine the actual site of proteolytic cleavage as shown in Figure 6. Peptide mapping by tryptophan-specific cleavage with BNPS-skatole is used to roughly determine the approximate position of cleavage in each molecule. Western blot analysis using SP antisera following BNPS-skatole treatment (Figure 6B) reveals fragments whose lengths of -10.5 and -9.5 kDa, corresponding to wild type and $BAP_{11\text{-}28}$respectively, confirming that cleavage occurs in the C-terminal portion of the PN-2-like protein as expected (Figure 6A). To determine the actual position of cleavage, secreted fragment is partially purified, treated with cyanogen bromide and relevant C-terminal peptides derived from APP-REP wild type.

DISCUSSION

[0069] The expression of a truncated form of APP-751, namely APP-REP 751 (pCLL602) is examined and its normal cleavage by secretase. Comparison of the nontransfected cells and those transfected with APP-REP 751, in both COS-1 transient and CHO stable expression systems, show the production of shorter secreted protein derived from APP-REP. Furthermore, upon a prolonged exposure of the fluorogram only one band is observed in condition medium. Epitope mapping with antibodies to N- and C-terminal domains of APP-REP and amino acid sequencing suggest post-translational cleavage at a site similar to that reported for intact APP protein and other truncated APP constructs similar to that reported for intact APP protein and other truncated APP constructs. Pulse-chase experiments reveal post-translational modifications, believed to be similar to those described for the intact APP protein, in which a single ~63 kDa product is chased up to ~76 kDa in the first 30 minutes. Appearance of the ~76 kDa cell membrane associated protein precedes the release of a ~67 kDa product into the CM. The released form, which is not observed in the cell lysate fraction, steadily accumulates in the conditioned medium well after the ~76 kDa band has begun to disappear suggesting a precursor-product relationship. These data indicate that the APP-REP protein is a good representation of the naturally occurring APP with respect to post-translational synthesis, processing, and stability in a tissue culture system.

[0070] Epitope mapping of APP-REP 751 mutants suggest that BAP_{E22Q}, as well as the $BAP_{\Delta 11\text{-}28}$deletion constructs, are initially expressed as larger proteins of predicted lengths which subsequently are cleaved to release N-terminal fragments into the CM. The pulse-chase experiments indicate the cell-associated and secreted forms accumulate with similar kinetics.

TABLE 1

Construction of APP-REP Partials

A. pSK(+) Amino-Terminal Constructs: Cloning of APP Isoform and Reporter Epitope (EcoRI-HindIII Fragments)

Plasmid Name	APP Isoform (EcoRI-XhoI Fragment)	Reporter Epitope (XhoI-HindIII Fragment)
pCLL983	APP-695	Substance P*
pCLL935	APP-751	Substance P
pCLL934	APP-770**	Substance P
pCLL913	APP-770#	Substance P

Notes:

* Substance P is a peptide containing 11 residues with the amino acid sequence of RPKPQQFFGLM.

** 5' untranslated sequences derived from the shorter APP-770 cDNA form.

# 5' untranslated sequences derived from the longer APP-751 cDNA form.

B. pSL301 Carboxy-Terminal Constructs: Cloning of BAP-Encoding APP Reporter Epitope Fusions (HindIII-BamHI/SalI Fragment)

Plasmid Name	Met-Enkephalin (ME) Fusion at end of:	Name of Variation
pCLL947	Full-Length APP	APP-BAP-APP-ME
pCLL914	Transmembrane Domain	APP-BAP-TM-ME
pCLL937	BAP	APP-BAP-ME

TABLE 1

Construction of APP-REP Partials

(Continued)

C. pSL301 Carboxy-Terminal Full-Length APP-ME Constructs: Introduction of Mutations in BAP (HindIII-BamHI/SalI Fragment)

Plasmid Name	Met-Enkephalin Fusion at End of:	Name of Variation
pCLL949	E to Q substitution at BAP aa#22	BAP E22Q
pCLL957	G to A substitution at BTaa#10, deletion of BAP AA#11-28 and creation of NdeI site	BAP-vaa11-28

TABLE 2

Assembly of APP-REP Full-Length Constructs Containing Substance P and Met-Enkephalin Reporter Epitopes and BAP or a Variation of BAP

Plasmid Name	Construct Name/Variation	Plasmid (N-Terminus)	Restriction Fragment (C-Terminus)
pCLL618	APP-REP-695	pCLL983	pCLL947
pCLL964	APP-REP-751	pCLL935	pCLL947
pCLL962	APP-REP-770	pCLL934	pCLL947
pCLL619	APP-REP-695/$\text{BAP}_{\text{E to Q}}$	pCLL983	pCLL949
pCLL989	APP-REP-751/$\text{BAP}_{\text{E to Q}}$	pCLL935	pCLL949
pCLL987	APP-REP-770/$\text{BAP}_{\text{E to Q}}$	pCLL934	pCLL949
pCLL620	APP-REP-695/$\text{BAP}/_{\Delta\text{aa11-28}}$	pCLL983	pCLL957
pCLL990	APP-REP-751/$\text{BAP}_{\Delta\text{aa11-28}}$	pCLL935	pCLL957
pCLL988	APP-REP-770/$\text{BAP}_{\Delta\text{aa11-28}}$	pCLL934	pCLL957

TABLE 3
Subcloning of APP-REP Full-Length Constructs and Human Growth Hormone (hGH) into pcDNA-1-Neo[XS]

Plasmid Name	Construct Name (in pcDNA-1-neo)	Source of Insert
pCLL600	pcDNA-1-neo-hGH	p0GH*
pCLL601	pcDNA-1-neo[XS]	Synthetic Fragment**
pCLL602	APP-REP-751	pCLL964
pCLL603#	APP-REP-751/BAP$_{E\ to\ Q}$	pCLL989
pCLL604#	APP-REP-751/BAP$_{\Delta aa11\text{-}28}$	pCLL990
pCLL605	APP-REP-770	pCLL962
pCLL606	APP-REP-770/BAP$_{E\ to\ Q}$	pCLL987
pCLL607	APP-REP-770/BAP$_{\Delta aa11\text{-}28}$	pCLL988

Notes:

* The HindIII-EcoRI (blunt-ended) fragment encoding hGH sequences of p0HG (Nichols Diagnostics) was subcloned into the HindIII-EcoRI (blunt-ended) sites of pcDNA-1-neo.

** The HindIII-XbaI fragment of the pcDNA-1-neo polylinker was replaced with a synthetic fragment which destroyed the original XbaI site and introduced several unique sites (HindIII-BamHI-XbaI-XhoI-SalI).

# Also created by an alternative strategy using the same pSK(+) plasmids.

TABLE 4
"Secretase-Minus" APP-REP Constructs Engineered by Oligonucleotide-Directed Mutagenesis

Plasmid Name	Mutation Identity	Mutated BAP Sequence Compared to Wild Type								Percent** Secretion	
		14	15	16	|	17	18	19	20		
pCLL602	BAP*	CAT	CAA	AAA	|	TTG	GTG	TTC	TTT	100	31844 E
		H	Q	K	|	L	V	F	F		
pCLL608	BAP-16KE	CAT	CAA	GAG	|	TTG	GTG	TTC	TTT	0	31844 F
		H	Q	E	|	L	V	F	F		
pCLL609	BAP-16KV	CAT	CAA	GTG	|	TTG	GTG	TTC	TTT	10-20	31844 G
		H	Q	V	|	L	V	F	F		
pCLL610	BAP-19FP	CAT	CAA	AAA	|	TTG	GTG	CCG	TTT	10-20	31844 H
		H	Q	K	|	L	V	P	F		

Notes:

* Wild-type BAP

* % secretion relative to wild type BAP sequence as determined by Sisodia.

TABLE 5
APP-REP Constructs Modeling APP Mutations Associated with Diseases Involving BAP Deposition

APP "717" MUTATIONS

// APP Transmembrane Domain /

// [BAP]

		711	712	713	714	715	716	717	718	719
		[40	41	42]						
pCLL602	APP*	GTC	ATA	GCG	ACA	GTG	ATC	GTC	ATC	ACC
		V	I	A	T	V	I	V	I	T
pCLL611	717VI**	GTC	ATA	GCG	ACA	GTG	ATC	ATC	ATC	ACC
		V	I	A	T	V	I	I	I	T
pCLL612	717VG@	GTC	ATA	GCG	ACA	GTG	ATC	GGC	ATC	ACC
		V	I	A	T	V	I	G	I	T
pCLL613	717VF$	GTC	ATA	GCG	ACA	GTG	ATC	TTC	ATC	ACC
		V	I	A	T	V	I	F	I	T

TABLE 5 (continued)

DUTCH DISEASE			:	V (secretase clip)						
	686	687	:	688	689	690	691	692	693	694
	[15	16	:	17	18	19	20	21	22	23]
pCLL602 BAP*	CAA	AAA	:	TTG	GTG	TTC	TTT	GCG	GAA	GAT
	Q	K	:	L	V	F	F	A	E	D
pCLL603 BAP-22EQ#	CAA	AAA	:	TTG	GTG	TTC	TTT	GCA	CAA	GAT
pCLL606#	Q	K	:	L	V	F	F	A	Q	D

Notes:

# APP-REP-751 and -770 derived BAP-22EQ constructs.

**** Goate et al. (1991) Nature, 349:704-706; Yoshioka et al. (1991) BBRC 178:1141-1146; Naruse et al. (1991) Lancet 337:978-979.**

@ Chartier-Harlin et al. (1991) Nature 353:844-846.

$ Murrell et al. (1991) Science 254:97-99.

Claims

1. A nucleic acid molecule encoding an amyloid precursor mutein, which comprises a nucleic acid sequence encoding at least one marker, the entire β-amyloid protein domain (BAP) or variants BAP_{E22Q} having an E to Q substitution at BAP amino acid #22 or $BAP_{\Delta aa11-28}$ and an amyloid precursor protein from which a block of 276 amino acids has been deleted.

2. The nucleic acid molecule of claim 1, wherein the nucleic acid molecule is a nucleic acid molecule selected from the group consisting of DNA, cDNA or RNA.

3. The nucleic acid molecule of claim 1, wherein the nucleic acid molecule is selected from the group consisting of pCLL964 (ATCC deposit no.: 68974) and pCLL602 (ATCC deposit no.: 68971).

4. The nucleic acid molecule of claim 1, wherein the amyloid precursor protein encoded by the nucleic acid sequence comprises 695, 751 or 770 amino acids prior to the deletion of the block of 276 amino acids.

5. A nucleic acid molecule encoding an amyloid precursor protein which comprises from the 5' end to the 3' end a nucleic acid sequence encoding a marker and an amyloid precursor protein comprising 695, 751 or 770 amino acids from which a block of 276 amino acids has been deleted, and which excludes the β-amyloid protein domain.

6. The nucleic acid molecule of claim 5, wherein the nucleic acid molecule is pCLL935 (ATCC deposit no.: 68972).

7. A nucleic acid molecule encoding an amyloid precursor protein which comprises from the 5' end to the 3' end a nucleic acid sequence encoding a marker and the β-amyloid protein domain variants BAP_{E22Q} having an E to Q substitution at BAP amino acid #22 or $BAP_{\Delta aa11\text{-}28}$.

8. A nucleic acid molecule encoding an amyloid precursor protein which comprises from the 5' end to the 3' end a nucleic acid sequence encoding Met-Enkephalin as a marker and the β-amyloid protein domain or variants BAP_{E22Q} having an E to Q substitution at BAP amino acid #22 or $BAP_{\Delta aa11\text{-}28}$

9. The nucleic acid molecule of claim 8, wherein the nucleic acid molecule is pCLL947 (ATCC deposit no.: 68973).

10. A vector comprising the nucleic acid molecule of claim 1, claim 5, claim 7 or claim 8.

11. A host cell stably transformed or transfected by a vector comprising the nucleic acid molecule of claim 1, claim 5, claim 7 or claim 8.

12. A recombinant polypeptide produced by the nucleic acid molecule of claim 1, claim 5, claim 7 or claim 8.

13. A method of detecting the presence of the recombinant polypeptide of claim 12 in a sample, comprising the steps of:

    (a) contacting an antibody directed to the marker and the sample under suitable conditions to favor the formation of an antibody-antigen complex, and
    (b) detecting the presence of any complex so formed.

14. A method of screening for a compound which inhibits or augments the formation of β-amyloid protein, comprising the steps of:

    (a) measuring the amount of marker in a suitable medium containing transfected cells stably or transiently expressing the nucleic acid molecule of claim 1,
    (b) treating said cells with the compound. and
    (c) testing the medium for an increase in the amount of the marker.

Patentansprüche

1. Nukleinsäuremolekül, das für ein Amyloidvorläufermutein kodiert, umfassend eine Nukleinsäuresequenz, die für mindestens einen Marker, für die gesamte β-Amyloidproteindomäne (BAP) oder deren Varianten BAP_{E22Q} mit einem E zu Q Austausch an BAP Aminosäure Nr. 22 oder $BAP_{\Delta aa11\text{-}28}$ und für ein Amyloidvorläuferprotein kodiert, von dem ein Block von 276 Aminosäuren deletiert wurde.

2. Das Nukleinsäuremolekül von Anspruch 1, das aus der Gruppe bestehend aus DNA, cDNA oder RNA ausgewählt ist.

3. Das Nukleinsäuremolekül von Anspruch 1, das aus der Gruppe bestehend aus pCLL964 (ATCC Hinterlegung Nr. 68974) und pCLL602 (ATCC Hinterlegung Nr. 68971) ausgewählt ist.

4. Das Nukleinsäuremolekül von Anspruch 1, wobei das von der Nukleinsäuresequenz kodierte Amyloidvorläuferprotein 695, 751 oder 770 Aminosäuren vor der Deletion des Blocks von 276 Aminosäuren umfasst.

5. Nukleinsäuremolekül, das für ein Amyloidvorläuferprotein kodiert, umfassend vom 5' zum 3' Ende eine Nukleinsäuresequenz, die für einen Marker und für ein Amyloidvorläuferprotein kodiert, das 695, 751 oder 770 Aminosäuren umfasst, von dem ein Block von 276 Aminosäuren deletiert wurde und das die β-Amyloidproteindomäne nicht umfasst.

6. Das Nukleinsäuremolekül von Anspruch 5, das pCLL935 (ATCC Hinterlegung Nr. 68972) ist.

7. Nukleinsäuremolekül, das für ein Amyloidvorläuferprotein kodiert, umfassend vom 5' zum 3' Ende eine Nukleinsäuresequenz, die für einen Marker und für die β-Amyioidproteindomän-Varianten BAP_{E22Q} mit einem E zu Q Austausch an BAP Aminosäure Nr. 22 oder $BAP_{\Delta aa11\text{-}28}$ kodiert.

8. Nukleinsäuremolekül, das für ein Amyloid-Vorläufer-Protein kodiert, umfassend vom 5' zum 3' Ende eine Nukleinsäuresequenz, die für Met-Enkephalin als Marker und für die β-Amyloidproteindomän-Varianten BAP_{E22Q} mit einem E zu Q Austausch an BAP Aminosäure Nr. 22 oder $BAP_{\Delta aa11\text{-}28}$ kodiert.

9. Das Nukleinsäuremolekül von Anspruch 8, das pCLL947 (ATCC Hinterlegung Nr. 68973) ist.

10. Vektor umfassend das Nukleinsäuremolekül von Anspruch 1, 5, 7 oder 8.

11. Wirtszelle, die mit einem Vektor umfassend das Nukleinsäuremolekül von Anspruch 1, 5, 7 oder 8 stabil transformiert oder transfiziert ist.

12. Rekombinantes Polypeptid, das mit dem Nukleinsäuremolekül von Anspruch 1, 5, 7 oder 8 hergestellt ist.

13. Verfahren zur Detektion der Anwesenheit des rekombinanten Polypeptids von Anspruch 12 in einer Probe, das die folgenden Schritte umfasst:

(a) Kontaktieren eines gegen den Marker und die Probe gerichteten Antikörpers unter geeigneten Bedingungen, um die Bildung eines Antikörper-Antigen-Komplexes zu begünstigen, und
(b) Detektieren eines so gebildeten Komplexes.

14. Verfahren zum Screening nach einer Verbindung, die die Bildung des β-Amyloidproteins inhibiert oder verstärkt, umfassend die Schritte:

(a) Messen der Markermenge in einem geeigneten Medium, das transfizierte Zellen enthält, die stabil oder transient das Nukleinsäuremolekül von Anspruch 1 exprimieren,
(b) Behandeln der Zellen mit der Verbindung und
(c) Testen des Mediums auf eine Zunahme der Markermenge.

Revendications

1. Molécule d'acide nucléique codant pour une mutéine précurseur d'amyloïde, comprenant une séquence d'acide nucléique codant pour au moins un marqueur, le domaine complet de la protéine amyloïde β (BAP) ou le variant BAP_{E22Q} comportant un remplacement de E par Q à l'aminoacide N° 22 de la protéine BAP ou $BAP_{\Delta aa11\text{-}28}$, et une protéine précurseur d'amyloïde dont un segment de 276 aminoacides a été supprimé.

2. Molécule d'acide nucléique selon la revendication 1, dans laquelle la molécule d'acide nucléique est une molécule d'acide nucléique choisie parmi un ADN, un ADNc ou un ARN.

3. Molécule d'acide nucléique selon la revendication 1, dans laquelle la molécule d'acide nucléique est choisie parmi pCLL964 (numéro de dépôt ATCC 68974) et pCLL602 (numéro de dépôt ATCC 68971).

4. Molécule d'acide nucléique selon la revendication 1, dans laquelle la protéine précurseur d'amyloïde codée par la séquence d'acide nucléique comprend 695, 751 ou 770 aminoacides avant la suppression du segment de 276 aminoacides.

5. Molécule d'acide nucléique codant pour une protéine précurseur d'amyloïde comprenant de l'extrémité 5' à l'extrémité 3', une séquence d'acide nucléique codant pour un marqueur et une protéine précurseur d'amyloïde comprenant 695, 751 ou 770 aminoacides dont un segment de 276 aminoacides a été supprimé, et qui exclut le domaine de la protéine amyloïde β.

6. Molécule d'acide nucléique selon la revendication 5, dans laquelle la molécule d'acide nucléique est pCLL935 (numéro de dépôt ATCC 68972).

7. Molécule d'acide nucléique codant pour une protéine précurseur d'amyloïde comprenant de l'extrémité 5' à l'extrémité 3', une séquence d'acide nucléique codant pour un marqueur et le variant de domaine de la protéine amyloïde β BAP_{E22Q} comportant un remplacement de E par Q à l'aminoacide N° 22 de la protéine BAP ou $BAP_{\Delta aa11\text{-}28}$.

8. Molécule d'acide nucléique codant pour une protéine précurseur d'amyloïde comprenant de l'extrémité 5' à l'extrémité 3', une séquence d'acide nucléique codant pour la mét-encéphaline en tant que marqueur, et le domaine de la protéine amyloïde β ou le variant BAP_{E22Q} comportant un remplacement de E par Q à l'aminoacide N° 22 de la protéine BAP, ou $BAP_{\Delta aa11\text{-}28}$.

9. Molécule d'acide nucléique selon la revendication 8, dans laquelle la molécule d'acide nucléique est pCLL947 (numéro de dépôt ATCC 68973).

10. Vecteur comprenant la molécule d'acide nucléique de la revendication 1, de la revendication 5, de la revendication 7 ou de la revendication 8.

11. Cellule hôte transformée ou transfectée de manière stable par un vecteur comprenant la molécule d'acide nucléique de la revendication 1, de la revendication 5, de la revendication 7 ou de la revendication 8.

12. Polypeptide recombinant produit par la molécule d'acide nucléique de la revendication 1, de la revendication 5, de la revendication 7 ou de la revendication 8.

13. Procédé de détection de la présence du polypeptide recombinant de la revendication 12 dans un échantillon, comprenant les étapes consistant :

(a) à mettre en contact un anticorps dirigé contre le marqueur et l'échantillon dans des conditions appropriées pour favoriser la formation d'un complexe anticorps-antigène, et
(b) à détecter la présence d'un complexe ainsi formé.

14. Procédé de sélection d'un composé empêchant ou augmentant la formation de la protéine amyloide β, comprenant les étapes consistant :

(a) à mesurer la quantité de marqueur dans un milieu approprié contenant des cellules transfectées de manière stable ou exprimant temporairement la molécule d'acide nucléique de la revendication 1,
(b) à traiter lesdites cellules avec le composé, et
(c) à tester le milieu à l'égard d'un accroissement de la quantité du marqueur.

Figure 1.

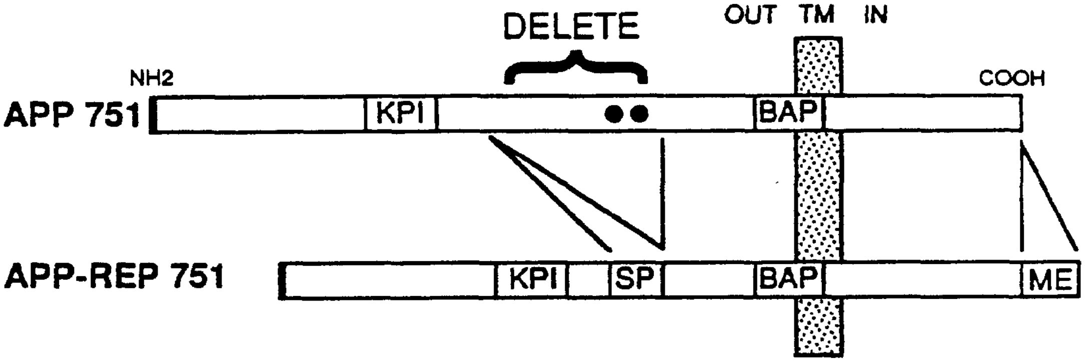

DELETE
OUT
TM
IN
NH2
COOH
APP 751
KPI
BAP
APP-REP 751
KPI
SP
BAP
ME

Figure 2.

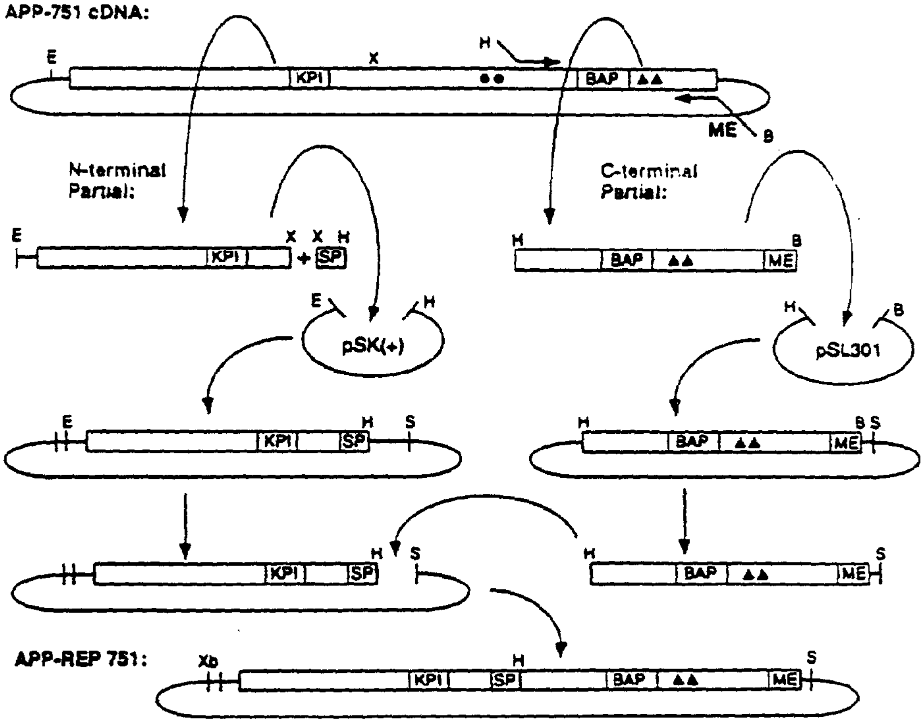

APP-751 cDNA:
E
X
H
KPI
BAP
ME
B
N-terminal Partial:
C-terminal Partial:
E
X
X
H
SP
+
H
B
E
H
pSK(+)
H
B
pSL301
E
H
S
H
B S
H
S
H
S
APP-REP 751:
Xb
H
S

Figure 3.

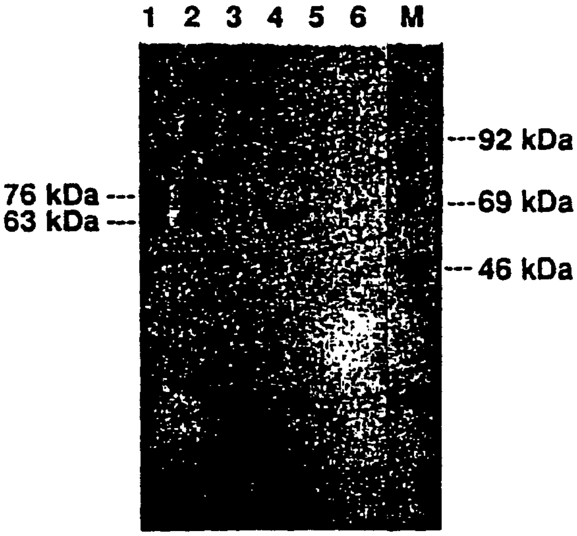


Figure 4.

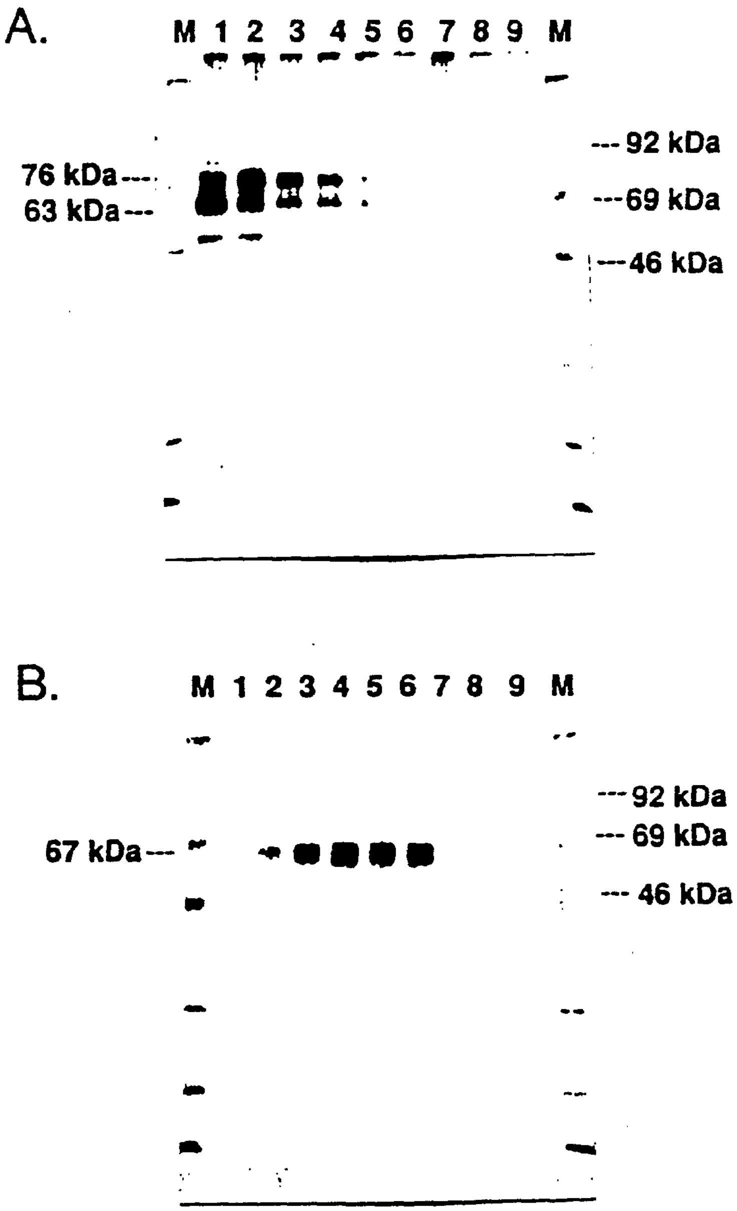

A.
M 1 2 3 4 5 6 7 8 9 M
76 kDa
63 kDa
92 kDa
69 kDa
46 kDa
B.
M 1 2 3 4 5 6 7 8 9 M
67 kDa
92 kDa
69 kDa
46 kDa

Figure 5.

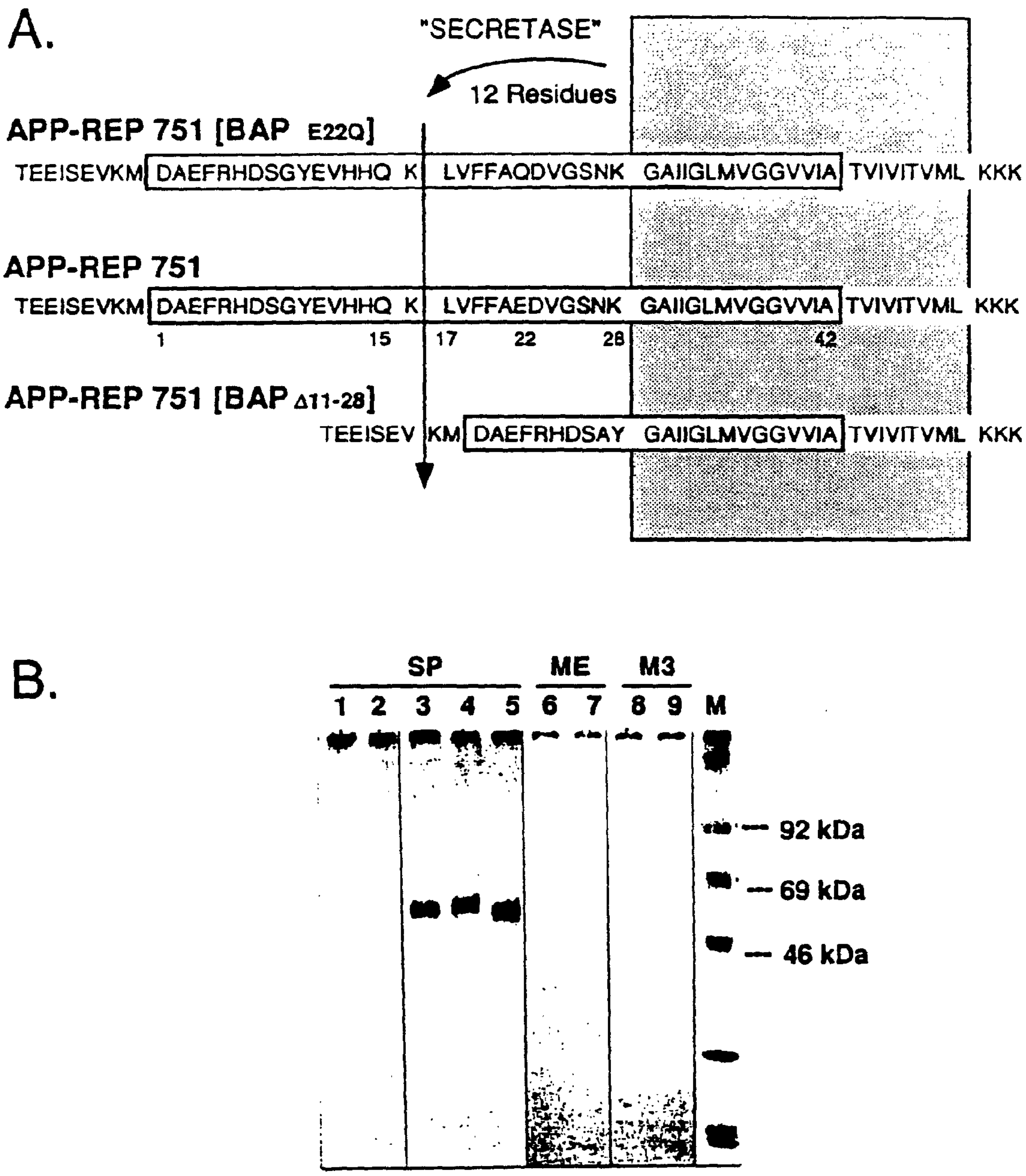
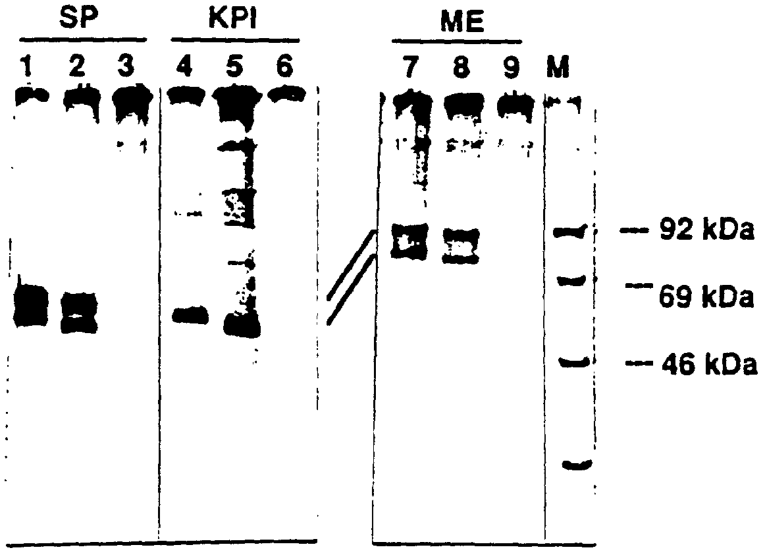

A.
"SECRETASE"
12 Residues
APP-REP 751 [BAP E22Q]
TEEISEVKM DAEFRHDSGYEVHHQ K LVFFAQDVGSNK GAIIGLMVGGVVIA TVIVITVML KKK
APP-REP 751
TEEISEVKM DAEFRHDSGYEVHHQ K LVFFAEDVGSNK GAIIGLMVGGVVIA TVIVITVML KKK
1
15
17
22
28
42
APP-REP 751 [BAP Δ11-28]
TEEISEV KM DAEFRHDSAY GAIIGLMVGGVVIA TVIVITVML KKK
B.
SP
ME
M3
1
2
3
4
5
6
7
8
9
M
92 kDa
69 kDa
46 kDa

Figure 5.

C.



D.

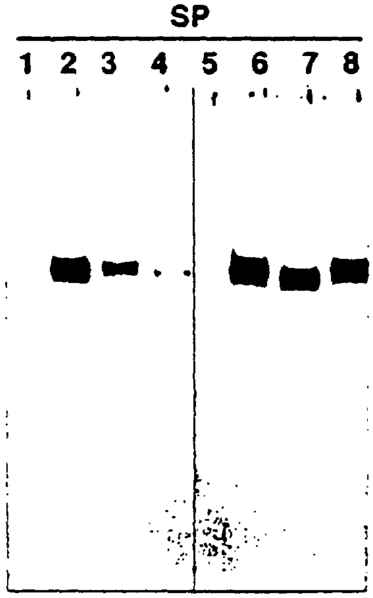


Figure 5.

E.

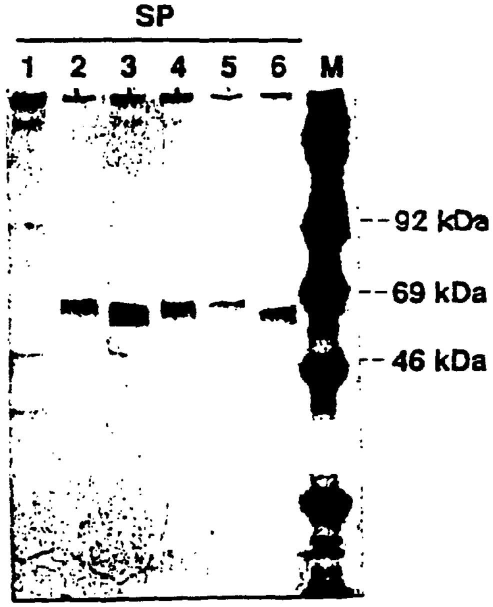


Figure 6.

A.

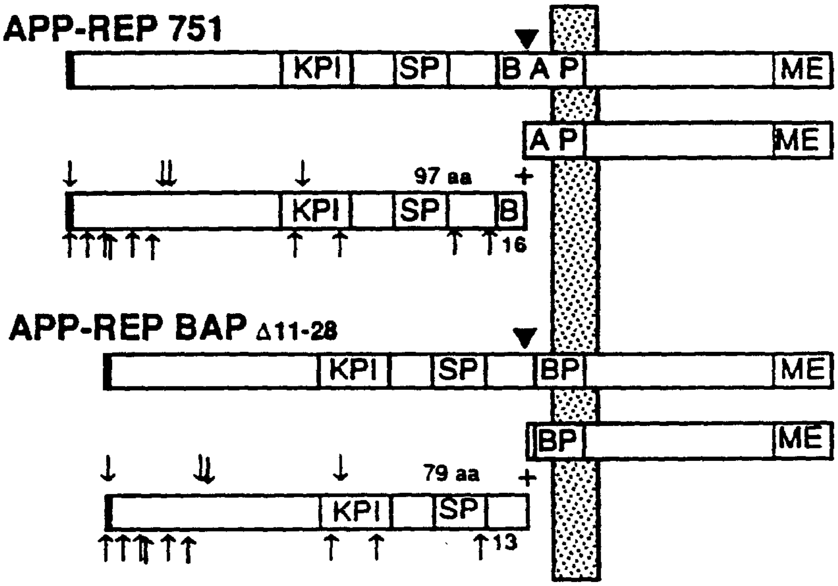
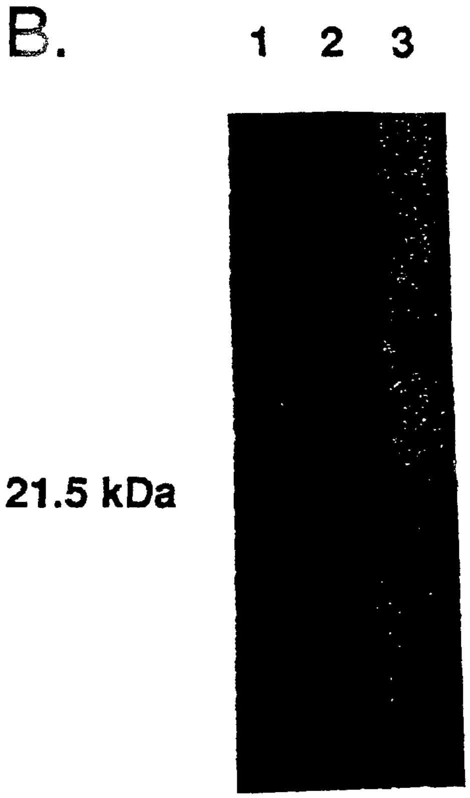

APP-REP 751
KPI
SP
B A P
ME
A P
ME
97 aa
KPI
SP
B
16
APP-REP BAP Δ11-28
KPI
SP
BP
ME
BP
ME
79 aa
KPI
SP
13

FIGURE 6.



```
SEQUENCE:  pCLL602  (APP-REP 751 protein)
VECTOR:    pcDNA-I-neo     (Invitrogen)
           pcDNA-I-neo-XS (JSJ modified polylinker to permit directional
              subcloning into XbaI-SalI sites)
INSERT:    XbaI-SalI fragment encoding APP-REP from pCLL964     16-1711
SEQUENCE:  5' polylinker:                                        2-47
              HindIII-XbaI from pcDNA-I-neo-XS                   2-15
              XbaI-EcoRI from pBluescript SK+                   16-47
           APP-REP 751:
              Amino-terminal partial from pCLL935):             48-1314
                 5' untranslated APP cDNA (from EcoRI)          48-195
                 N-terminal APP (to XhoI)                      196-1273
                 Substance P marker (XhoI to HindIII)         1274-1314
              Carboxy-terminal partial from pCLL947):         1314-1671
                 C-terminal APP and BAP (from novel HindIII)  1314-1656
                 Met-enkephalin marker (plus stop codon)      1657-1674
           3' polylinker:
              BamHI-SalI from pSL301                          1674-1711
              SalI-end of sequence from pcDNA-I-neo-XS        1712-1721
```

```
        10         20         30         40         50
         *          *          *          *          *
AAGCTTGGGG ATCCGCTCTA GAACTAGTGG ATCCCCCGGG CTGCAGGAAT
TTCGAACCCC TAGGCGAGAT CTTGATCACC TAGGGGGCCC GACGTCCTTA

        60         70         80         90        100
         *          *          *          *          *
TCGGGGGGGG CAGCGGTAGG CGAGAGCACG CGGAGGAGCG TGCGCGGGGC
AGCCCCCCCC GTCGCCATCC GCTCTCGTGC GCCTCCTCGC ACGCGCCCCG

       110        120        130        140        150
         *          *          *          *          *
CCCGGGAGAC GGCGGCGGTG GCGGCGCGGG CAGAGCAAGG ACGCGGCGGA
GGGCCCTCTG CCGCCGCCAC CGCCGCGCCC GTCTCGTTCC TGCGCCGCCT

       160        170        180        190
         *          *          *          *
TCCCACTCGC ACAGCAGCGC ACTCGGTGCC CCGCGCAGGG TCGCG
AGGGTGAGCG TGTCGTCGCG TGAGCCACGG GGCGCGTCCC AGCGC

   200          210           220          230          240
     *            *             *            *            *
ATG CTG CCC GGT TTG GCA CTG CTC CTG CTG GCC GCC TGG ACG GCT
TAC GAC GGG CCA AAC CGT GAC GAG GAC GAC CGG CGG ACC TGC CGA
Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala>

          250          260          270           280
            *            *            *             *
CGG GCG CTG GAG GTA CCC ACT GAT GGT AAT GCT GGC CTG CTG GCT GAA
GCC CGC GAC CTC CAT GGG TGA CTA CCA TTA CGA CCG GAC GAC CGA CTT
Arg Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu>
```

FIGURE 7

```
290          300          310           320          330
  *            *            *             *            *
  CCC CAG ATT GCC ATG TTC TGT GGC AGA CTG AAC ATG CAC ATG AAT GTC
  GGG GTC TAA CGG TAC AAG ACA CCG TCT GAC TTG TAC GTG TAC TTA CAG
  Pro Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val>

  340           350          360          370           380
    *             *            *            *             *
  CAG AAT GGG AAG TGG GAT TCA GAT CCA TCA GGG ACC AAA ACC TGC ATT
  GTC TTA CCC TTC ACC CTA AGT CTA GGT AGT CCC TGG TTT TGG ACG TAA
  Gln Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile>

     390          400           410          420          430
       *            *             *            *            *
  GAT ACC AAG GAA GGC ATC CTG CAG TAT TGC CAA GAA GTC TAC CCT GAA
  CTA TGG TTC CTT CCG TAG GAC GTC ATA ACG GTT CTT CAG ATG GGA CTT
  Asp Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu>

        440          450          460           470          480
          *            *            *             *            *
  CTG CAG ATC ACC AAT GTG GTA GAA GCC AAC CAA CCA GTG ACC ATC CAG
  GAC GTC TAG TGG TTA CAC CAT CTT CGG TTG GTT GGT CAC TGG TAG GTC
  Leu Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln>

          490           500          510          520
            *             *            *            *
  AAC TGG TGC AAG CGG GGC CGC AAG CAG TGC AAG ACC CAT CCC CAC TTT
  TTG ACC ACG TTC GCC CCG GCG TTC GTC ACG TTC TGG GTA GGG GTG AAA
  Asn Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe>

530          540          550           560          570
  *            *            *             *            *
  GTG ATT CCC TAC CGC TGC TTA GTT GGT GAG TTT GTA AGT GAT GCC CTT
  CAC TAA GGG ATG GCG ACG AAT CAA CCA CTC AAA CAT TCA CTA CGG GAA
  Val Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu>

  580           590          600          610           620
    *             *            *            *             *
  CTC GTT CCT GAC AAG TGC AAA TTC TTA CAC CAG GAG AGG ATG GAT GTT
  GAG CAA GGA CTG TTC ACG TTT AAG AAT GTG GTC CTC TCC TAC CTA CAA
  Leu Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val>

     630          640           650          660          670
       *            *             *            *            *
  TGC GAA ACT CAT CTT CAC TGG CAC ACC GTC GCC AAA GAG ACA TGC AGT
  ACG CTT TGA GTA GAA GTG ACC GTG TGG CAG CGG TTT CTC TGT ACG TCA
  Cys Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser>

        680          690          700           710          720
          *            *            *             *            *
  GAG AAG AGT ACC AAC TTG CAT GAC TAC GGC ATG TTG CTG CCC TGC GGA
  CTC TTC TCA TGG TTG AAC GTA CTG ATG CCG TAC AAC GAC GGG ACG CCT
  Glu Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly>
```

FIGURE 7
(continued)

```
          730         740         750         760
            *           *           *           *
ATT GAC AAG TTC CGA GGG GTA GAG TTT GTG TGT TGC CCA CTC GCT GAA
TAA CTG TTC AAG GCT CCC CAT CTC AAA CAC ACA ACG GGT GAG CGA CTT
Ile Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu>

770         780         790         800         810
 *           *           *           *           *
GAA AGT GAC AAT GTG GAT TCT GCT GAT GCG GAG GAG GAT GAC TCG GAT
CTT TCA CTG TTA CAC CTA AGA CGA CTA CGC CTC CTC CTA CTG AGC CTA
Glu Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp>

  820         830         840         850         860
    *           *           *           *           *
GTC TGG TGG GGC GGA GCA GAC ACA GAC TAT GCA GAT GGG AGT GAA GAC
CAG ACC ACC CCG CCT CGT CTG TGT CTG ATA CGT CTA CCC TCA CTT CTG
Val Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp>

    870         880         890         900         910
      *           *           *           *           *
AAA GTA GTA GAA GTA GCA GAG GAG GAA GAA GTG GCT GAG GTG GAA GAA
TTT CAT CAT CTT CAT CGT CTC CTC CTT CTT CAC CGA CTC CAC CTT CTT
Lys Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu>

       920         930         940         950         960
         *           *           *           *           *
GAA GAA GCC GAT GAT GAC GAG GAC GAT GAG GAT GGT GAT GAG GTA GAG
CTT CTT CGG CTA CTA CTG CTC CTG CTA CTC CTA CCA CTA CTC CAT CTC
Glu Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu>

          970         980         990        1000
            *           *           *           *
GAA GAG GCT GAG GAA CCC TAC GAA GAA GCC ACA GAG AGA ACC ACC AGC
CTT CTC CGA CTC CTT GGG ATG CTT CTT CGG TGT CTC TCT TGG TGG TCG
Glu Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser>

1010        1020        1030        1040        1050
   *           *           *           *           *
ATT GCC ACC ACC ACC ACC ACC ACC ACA GAG TCT GTG GAA GAG GTG GTT
TAA CGG TGG TGG TGG TGG TGG TGG TGT CTC AGA CAC CTT CTC CAC CAA
Ile Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val>

  1060        1070        1080        1090        1100
     *           *           *           *           *
CGA GAG GTG TGC TCT GAA CAA GCC GAG ACG GGG CCG TGC CGA GCA ATG
GCT CTC CAC ACG AGA CTT GTT CGG CTC TGC CCC GGC ACG GCT CGT TAC
Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met>

    1110        1120        1130        1140        1150
       *           *           *           *           *
ATC TCC CGC TGG TAC TTT GAT GTG ACT GAA GGG AAG TGT GCC CCA TTC
TAG AGG GCG ACC ATG AAA CTA CAC TGA CTT CCC TTC ACA CGG GGT AAG
Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe>
```

FIGURE 7
(continued)

```
        1160         1170          1180         1190         1200
           *            *             *            *            *
  TTT TAC GGC GGA TGT GGC GGC AAC CGG AAC AAC TTT GAC ACA GAA GAG
  AAA ATG CCG CCT ACA CCG CCG TTG GCC TTG TTG AAA CTG TGT CTT CTC
  Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu>

           1210         1220         1230          1240
              *            *            *             *
  TAC TGC ATG GCC GTG TGT GGC AGC GCC ATT CCT ACA ACA GCA GCC AGT
  ATG ACG TAC CGG CAC ACA CCG TCG CGG TAA GGA TGT TGT CGT CGG TCA
  Tyr Cys Met Ala Val Cys Gly Ser Ala Ile Pro Thr Thr Ala Ala Ser>

1250         1260          1270         1280         1290
   *            *             *            *            *
  ACC CCT GAT GCC GTT GAC AAG TAT CTC GAG CGG CCC AAG CCC CAG CAG
  TGG GGA CTA CGG CAA CTG TTC ATA GAG CTC GCC GGG TTC GGG GTC GTC
  Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Arg Pro Lys Pro Gln Gln>

   1300         1310         1320          1330         1340
      *            *            *             *            *
  TTC TTT GGC CTG ATG GGA AGC TTG ACA AAT ATC AAG ACG GAG GAG ATC
  AAG AAA CCG GAC TAC CCT TCG AAC TGT TTA TAG TTC TGC CTC CTC TAG
  Phe Phe Gly Leu Met Gly Ser Leu Thr Asn Ile Lys Thr Glu Glu Ile>

     1350          1360         1370         1380          1390
        *             *            *            *             *
  TCT GAA GTG AAG ATG GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA
  AGA CTT CAC TTC TAC CTA CGT CTT AAG GCT GTA CTG AGT CCT ATA CTT
  Ser Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu>

        1400         1410          1420         1430         1440
           *            *             *            *            *
  GTT CAT CAT CAA AAA TTG GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC
  CAA GTA GTA GTT TTT AAC CAC AAG AAA CGT CTT CTA CAC CCA AGT TTG
  Val His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn>

           1450         1460         1470          1480
              *            *            *             *
  AAA GGT GCA ATC ATT GGA CTC ATG GTG GGC GGT GTT GTC ATA GCG ACA
  TTT CCA CGT TAG TAA CCT GAG TAC CAC CCG CCA CAA CAG TAT CGC TGT
  Lys Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr>

1490         1500          1510         1520         1530
   *            *             *            *            *
  GTG ATC GTC ATC ACC TTG GTG ATG CTG AAG AAG AAA CAG TAC ACA TCC
  CAC TAG CAG TAG TGG AAC CAC TAC GAC TTC TTC TTT GTC ATG TGT AGG
  Val Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser>

   1540         1550         1560          1570         1580
      *            *            *             *            *
  ATT CAT CAT GGT GTG GTG GAG GTT GAC GCC GCT GTC ACC CCA GAG GAG
  TAA GTA GTA CCA CAC CAC CTC CAA CTG CGG CGA CAG TGG GGT CTC CTC
  Ile His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu>
```

FIGURE 7
(continued)

```
      90            1600           1610           1620            1630
       *              *              *              *               *
CGC CAC CTG TCC AAG ATG CAG CAG AAC GGC TAC GAA AAT CCA ACT TAC
GCG GTG GAC AGG TTC TAC GTC GTC TTG CCG ATG CTT TTA GGT TGG ATG
Arg His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr>

       1640           1650            1660           1670          1680
          *              *               *              *             *
AAG TTC TTT GAG CAG ATG CAG AAC TAT GGG GGC TTC ATG TAG GATCCA
TTC AAG AAA CTC GTC TAC GTC TTG ATA CCC CCG AAG TAC ATC CTAGGT
Lys Phe Phe Glu Gln Met Gln Asn Tyr Gly Gly Phe Met ***

       1690        1700        1710          1720
          *           *           *             *
TATATAGGGC CCGGGTTAT AATTACCTCA GGTCGACCTA GA
ATATATCCCG GGCCCAATA TTAATGGAGT CCAGCTGGAT CT
```

FIGURE 7
(continued)

```
Total of 12 files.
 RMRD3>TYPE APP770.
FOCUS        HUMAFPA4      3353 bp ss-mRNA          PRI       15-JUN-1989
Human amyloid A4 mRNA, complete cds.
ACCESSION    Y00264
KEYWORDS     amyloid fibril protein; cell surface glycoprotein.
SOURCE       human (Homo sapiens).
  ORGANISM   Homo sapiens
             Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia;
             Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae.
REFERENCE    1  (bases 1 to 3353; enum. -146 to 3207, no zero)
  AUTHORS    Kang,J., Lemaire,H.G., Unterbeck,A., Salbaum,J.M., Masters,C.L.,
             Grzeschik,K.-H., Multhaup,G., Beyreuther,K. and Mueller-Hill,B.
  TITLE      The precursor of Alzheimer's disease amyloid A4 protein resembles a
             cell-surface receptor
  JOURNAL    Nature 325, 733-736 (1987)
  STANDARD   simple automatic
REFERENCE    2  (bases 1 to 3353; enum. 1 to 3353)
  AUTHORS    Mueller Hill,B.
  JOURNAL    Unpublished (1987) Submitted to the EMBL data library.
  STANDARD   simple automatic
COMMENT      *source: tissue=cortex of brain; *source: developmental
             stage=5-month-old aborted fetus;
                EMBL features not translated to GenBank features:
                key         from     to        description
                SITE        3080    3085       polyA signal
                SITE        3089    3094       polyA signal
                SITE        3331    3336       polyA signal
                POLYA       3353    3353       polyA site
FEATURES        from  to/span       description
    pept         147     2234       amyloid A4 /nomgen="APP" /map="21q21.2"
                                    /hgml_locus_uid="LG0136J"
BASE COUNT       922 a     745 c     867 g     819 t
ORIGIN

      App770.  Length: 3353  May 29, 1990  11:30  Check: 6510  ..

        1  AGTTTCCTCG GCAGCGGTAG GCGAGAGCAC GCGGAGGAGC GTGCGCGGGG

       51  CCCCGGGAGA CGGCGGCGGT GGCGGCGCGG GCAGAGCAAG GACGCGGCGG

      101  ATCCCACTCG CACAGCAGCG CACTCGGTGC CCCGCGCAGG GTCGCGATGC

      151  TGCCCGGTTT GGCACTGCTC CTGCTGGCCG CCTGGACGGC TCGGGCGCTG

      201  GAGGTACCCA CTGATGGTAA TGCTGGCCTG CTGGCTGAAC CCCAGATTGC

      251  CATGTTCTGT GGCAGACTGA ACATGCACAT GAATGTCCAG AATGGGAAGT

      301  GGGATTCAGA TCCATCAGGG ACCAAAACCT GCATTGATAC CAAGGAAGGC

      351  ATCCTGCAGT ATTGCCAAGA AGTCTACCCT GAACTGCAGA TCACCAATGT

      401  GGTAGAAGCC AACCAACCAG TGACCATCCA GAACTGGTGC AAGCGGGGCC

      451  GCAAGCAGTG CAAGACCCAT CCCCACTTTG TGATTCCCTA CCGCTGCTTA

      501  GTTGGTGAGT TTGTAAGTGA TGCCCTTCTC GTTCCTGACA AGTGCAAATT

      551  CTTACACCAG GAGAGGATGG ATGTTTGCGA AACTCATCTT CACTGGCACA

      601  CCGTCGCCAA AGAGACATGC AGTGAGAAGA GTACCAACTT GCATGACTAC

      651  GGCATGTTGC TGCCCTGCGG AATTGACAAG TTCCGAGGGG TAGAGTTTGT

      701  GTGTTGCCCA CTGGCTGAAG AAAGTGACAA TGTGGATTCT GCTGATGCGG
```

FIGURE 8

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 751  AGGAGGATGA CTCGGATGTC TGGTGGGGCG GAGCAGACAC AGA TATGCA
 801  GATGGGAGTG AAGACAAAGT AGTAGAAGTA GCAGAGGAGG AAGAAGTGGC
 851  TGAGGTGGAA GAAGAAGAAG CCGATGATGA CGAGGACGAT GAGGATGGTG
 901  ATGAGGTAGA GGAAGAGGCT GAGGAACCCT ACGAAGAAGC CACAGAGAGA
 951  ACCACCAGCA TTGCCACCAC CACCACCACC ACCACAGAGT CTGTGGAAGA
1001  GGTGGTTCGA GTTCCTACAA CAGCAGCCAG TACCCCTGAT GCCGTTGACA
1051  AGTATCTCGA GACACCTGGG GATGAGAATG AACATGCCCA TTTCCAGAAA
1101  GCCAAAGAGA GGCTTGAGGC CAAGCACCGA GAGAGAATGT CCCAGGTCAT
1151  GAGAGAATGG GAAGAGGCAG AACGTCAAGC AAAGAACTTG CCTAAAGCTG
1201  ATAAGAAGGC AGTTATCCAG CATTTCCAGG AGAAAGTGGA ATCTTTGGAA
1251  CAGGAAGCAG CCAACGAGAG ACAGCAGCTG GTGGAGACAC ACATGGCCAG
1301  AGTGGAAGCC ATGCTCAATG ACCGCCGCCG CCTGGCCCTG GAGAACTACA
1351  TCACCGCTCT GCAGGCTGTT CCTCCTCGGC CTCGTCACGT GTTCAATATG
1401  CTAAAGAAGT ATGTCCGCGC AGAACAGAAG GACAGACAGC ACACCCTAAA
1451  GCATTTCGAG CATGTGCGCA TGGTGGATCC CAAGAAAGCC GCTCAGATCC
1501  GGTCCCAGGT TATGACACAC CTCCGTGTGA TTTATGAGCG CATGAATCAG
1551  TCTCTCTCCC TGCTCTACAA CGTGCCTGCA GTGGCCGAGG AGATTCAGGA
1601  TGAAGTTGAT GAGCTGCTTC AGAAAGAGCA AAACTATTCA GATGACGTCT
1651  TGGCCAACAT GATTAGTGAA CCAAGGATCA GTTACGGAAA CGATGCTCTC
1701  ATGCCATCTT TGACCGAAAC GAAAACCACC GTGGAGCTCC TTCCCGTGAA
1751  TGGAGAGTTC AGCCTGGACG ATCTCCAGCC GTGGCATTCT TTTGGGGCTG
1801  ACTCTGTGCC AGCCAACACA GAAAACGAAG TTGAGCCTGT TGATGCCCGC
1851  CCTGCTGCCG ACCGAGGACT GACCACTCGA CCAGGTTCTG GGTTGACAAA
1901  TATCAAGACG GAGGAGATCT CTGAAGTGAA GATGGATGCA GAATTCCGAC
1951  ATGACTCAGG ATATGAAGTT CATCATCAAA AATTGGTGTT CTTTGCAGAA
2001  GATGTGGGTT CAAACAAAGG TGCAATCATT GGACTCATGG TGGGCGGTGT
2051  TGTCATAGCG ACAGTGATCG TCATCACCTT GGTGATGCTG AAGAAGAAAC
2101  AGTACACATC CATTCATCAT GGTGTGGTGG AGGTTGACGC CGCTGTCACC
2151  CCAGAGGAGC GCCACCTGTC CAAGATGCAG CAGAACGGCT ACGAAAATCC
2201  AACCTACAAG TTCTTTGAGC AGATGCAGAA CT

                                         AGACCCCC GCCACAGCAG
2251  CCTCTGAAGT TGGACAGCAA AACCATTGCT TCACTACCCA TCGGTGTCCA
```

FIGURE 8
(continued)

2301 TTTATAGAAT AATGTGCGAA GAAACAAACC CGTTTTATGA TTTACTCATT

2351 ATCGCCTTTT GACAGCTGTG CTGTAACACA AGTACATGCC TGAACTTGAA

2401 TTAATCCACA CATCAGTAAT GTATTCTATC TCTCTTTACA TCTTGGTCTC

2451 TATACTACAT TATTAATGGG TTTTGTGTAC TGTAAAGAAT TTAGCTGTAT

2501 CAAACTAGTG CATGAATAGA TTCTCTCCTG ATTATTTATC ACATAGCCCC

2551 TTAGCCAGTT GTATATTATT CTTGTGGTTT GTGACCCAAT TAAGTCCTAC

2601 TTTACATATG CTTTAAGAAT CGATGGGGGA TGCTTCATGT GAACGTGGGA

2651 GTTCAGCTGC TTCTCTTGCC TAAGTATTCC TTTCCTGATC ACTATGCATT

2701 TTAAAGTTAA ACATTTTTAA GTATTTCAGA TGCTTTAGAG AGATTTTTTT

2751 TCCATGACTG CATTTTACTG TACAGATTGC TGCTTCTGCT ATATTTGTGA

2801 TATAGGAATT AAGAGGATAC ACACGTTTGT TTCTTCGTGC CTGTTTTATG

2851 TGCACACATT AGGCATTGAG ACTTCAAGCT TTTCTTTTTT TGTCCACGTA

2901 TCTTTGGGTC TTTGATAAAG AAAAGAATCC CTGTTCATTG TAAGCACTTT

2951 TACGGGGCGG GTGGGGAGGG GTGCTCTGCT GGTCTTCAAT TACCAAGAAT

3001 TCTCCAAAAC AATTTTCTGC AGGATGATTG TACAGAATCA TTGCTTATGA

3051 CATGATCGCT TTCTACACTG TATTACATAA ATAAATTAAA TAAAATAACC

3101 CCGGGCAAGA CTTTTCTTTG AAGGATGACT ACAGACATTA AATAATCGAA

3151 GTAATTTTGG GTGGGGAGAA GAGGCAGATT CAATTTTCTT TAACCAGTCT

3201 GAAGTTTCAT TTATGATACA AAAGAAGATG AAAATGGAAG TGGCAATATA

3251 AGGGGATGAG GAAGGCATGC CTGGACAAAC CCTTCTTTTA AGATGTGTCT

3301 TCAATTTGTA TAAAATGGTG TTTTCATGTA AATAAATACA TTCTTGGAGG

3351 AGC

RMRD3>

FIGURE 8
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(54) INDUCING ANTIBODY RESPONSE AGAINST SELF-PROTEINS WITH THE AID OF FOREIGN T-CELL EPITOPES

DIE INDUKTION DER ANTIKÖRPERANTWORT GEGEN EIGENE PROTEINE MIT HILFE FREMDER T-ZELLEPITOPE

PROCEDE D'INDUCTION DE REACTIONS IMMUNITAIRES CONTRE LES PROTEINES ENDOGENES A L'AIDE D'EPITOPES DE LYMPHOCYTES T EXOGENES

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Description

Field of the invention

This invention concerns a novel method for utilizing the immune apparatus to remove and/or down-regulate self-proteins, the presence of which somehow is unwanted in the individual. These could be proteins which are causing disease and/or other undesirable symptoms or signs of disease. Such proteins are removed by circulating autoantibodies which specifically are induced by vaccination. This invention describes a method for developing such autovaccines.

Background of the invention

Physiologically, the vertebrate immune system serves as a defense mechanism against invasion of the body by infectious objects such as micro-organisms. Foreign proteins are effectively removed via the reticuloendothelial system by highly specific circulating antibodies, and viruses and bacteria are attacked by a complex battery of cellular and humoral mechanisms including antibodies, cytotoxic T lymphocytes, Natural Killer cells, complement etc. The leader of this battle is the T helper (T_H) lymphocyte which, in collaboration with the Antigen Presenting Cells (APC), regulate the immune defense via a complex network of cytokines.

T_H lymphocytes recognize protein antigens presented on the surface of the APC. They do not recognize, however, native antigen per se. Instead, they appear to recognize a complex ligand consisting of two components, a "processed" (fragmented) protein antigen (the so-called T cell epitope) and a Major Histocompatibility Complex class II molecule (O. Werdelin et al., Imm. Rev. 106, 181 (1988)). This recognition eventually enables the T_H lymphocyte specifically to help B lymphocytes to produce specific antibodies towards the intact protein antigen (Werdelin et al., supra). A given T cell only recognizes a certain antigen-MHC combination and will not recognize the same or another antigen presented by a gene product of another MHC allele. This phenomenon is called MHC restriction.

Normally the individual's own proteins (the so-called self- or autoproteins) are not attacked by the immune apparatus. The described events thus generally are beneficial to the individual, but in rare cases the process goes wrong, and the immune system turns towards the individual's own components, which may lead to an autoimmune disease.

The presence of some self-proteins is inexpedient in situations where they, in elevated levels, induce disease symptoms. High levels of immunoglobulins of the IgE class are e.g. known to be important for the induction of type I allergy, and tumor necrosis factor I (TNFα) is known to be able to cause cachexia in cancer patients and patients suffering from other chronic diseases (H.N. Langstein et al., Cancer Res. 51, 2302-2306, 1991). TNFα also plays important roles in the inflammatory process (W.P. Arend et al., Arthritis Rheum. 33, 305-315, 1990). Hormones in sex-hormone dependent cancer are other examples of proteins which are unwanted in certain situations. There is therefore a need for the provision of a method for the development of autovaccines against such self-proteins.

Fragments of self-proteins are also presented by the APC, but normally such fragments are ignored or not recognized by the T helper lymphocytes. This is the main reason why individuals generally do not harbor autoantibodies in their serum.

It is, however, possible artificially to induce antibodies against self-proteins. This can be done, as previously mentioned, by covalent conjugation of the self-protein to an appropriate large foreign carrier protein as e.g. tetanus toxoid or key-hole limpet hemocyanin (KLH). Talwar et al. (G.P. Talwar et al, Int. J. Immunopharmacol. 14, 511-514, 1992) have been able to prevent reproduction in women using a vaccine consisting of a conjugate of human chorionic gonadotropin and tetanus toxoid. There are also other examples of such autoimmunogenic conjugates which have been used therapeutically in man and in animal models (D.R. Stanworth et al., Lancet 336, 1279-1281 (1990)). During the processing of such conjugates in the APC, the necessary T_H lymphocyte stimulatory epitopes are provided from the foreign protein eventually leading to the induction of antibodies against the self-protein as well as against the carrier protein. One disadvantage of using this principle is, however, that the antibody response towards the self-protein will be limited due to shielding of epitopes by the covalently linked carrier protein. Another disadvantage is the increased risk of inducing allergic side-effects due to the contemporary induction of an undesired very strong antibody response against the foreign carrier protein.

Other researchers have conjugated a single peptide predicted to be a T cell epitope chemically as a carrier to a self-peptide [D-Lys^6]GnRH which is a decapeptide acting as a hapten and managed to induce an autoantibody response with MHC restriction to that particular T cell epitope (S. Sad et al., Immunology 76, 599-603, 1992). This method seems to be more effective compared with conjugation to large carrier proteins. However, it will only induce antibodies in a population expressing the appropriate MHC molecules. This means that a rather large number of different T cell epitopes has to be conjugated to the self-peptide which will eventually disturb the B cell epitopes on the surface of the self-peptide. Extensive conjugation of proteins may furthermore have the opposite effect with regard to immunogenicity (international patent application No. WO 87/00056) and the surface exposed peptide T cell epitopes may be destroyed by proteolytic enzymes during antigen processing (S. Mouritsen, Scand. J. Immunol. 30, 723, 1989), making that method inexpedient. Also, the exact structure of such multi-conjugated self-peptides will not be chemically and pharmaceutically well-defined.

Recently an improved method has been proposed for breaking the B cell autotolerance by chemical conjugation of B and optionally also peptide T cell epitopes to a high molecular weight dextran molecule (WO 93/23076 published November 25, 1993). The disadvantages mentioned above, however, also hold true for said method.

Löwenadler et al, (Medline Accession No. 9208571 & Mol Immunol 29 1992 1185-1190; discloses chimeric proteins containing one or more copies of heterologous T helper epitopes, in particular ovalbumin 323-339 (ova), inserted at different positions. The chimeric proteins comprise one or more ova sequences flanked by the heat-stable enterotoxin of E. coli and the nine C-terminal amino acids of human insulin-like growth factor 1 (IGF-1), together with a synthetic analogue of the IgG-binding domain of protein A, the latter to aid the purification by means of IgG-Sepharone ™ affinity chromatography. Thus the chimeric proteins are fusion constructs of several different proteins. The effect of the presence of the ova sequences on the production of antibodies to the enterotoxin and the IGF-1 peptide was investigated in mice, i.e. the proteins used are not self-proteins.

WO-A-93/05810, Hellman, discusses possible mutations of protein and it is suggested that "heavily mutated" forms of the CH2-CH3 domains of IgE (a self-protein) may be used in a vaccine, these being created by exchange, deletion or insertion of amino acids in the sequence. This would create T-cell epitopes within the amino acid sequence, allowing the CH2-CH3 domains to act as an antigen per se and thus obviating the need to couple the protein to a carrier. "Slightly mutated" forms, according to the disclosure of Hellman, still need to be coupled to a suitable carrier protein in order to act as an antigen.

It has been suggested previously that a universally recognized strong T cell epitope could be associated with a foreign peptide having an antigenic structure representing a B-cell epitope using recombinant DNA technology (EP-A2-0 343 460) It has also been suggested to use peptidyl resin conjugates comprising an immunogenic or antigenic peptide incorporating a helper T-cell (T_H lymphocyte) reactive epitope and preferably a B-cell reactive epitope in the preparation of immunogenic compositions, e.g. vaccines. The conjugates are prepared by solid phase synthesis, preferably on a polyamide resin. (WO 90/15627). While the intent is to increase an antibody response towards the peptides in question, it has not been proposed that this could be done with the purpose of breaking the autotolerance of the immune system and induce an antibody response against self-proteins. Using these methods for induction of autoantibodies against self-proteins one a priori would expect the same rules to be true with regard to the above-mentioned limitations of the MHC restriction of the response. Surprisingly, however, by modulation of self-proteins using the method according to the invention, wherein a self-protein analog is produced by substitution of one or more peptide fragments by a corresponding number of peptides known to contain immunodominant T-cell epitopes, said substitution being carried out so as to essentially preserve the overall tertiary structure of the original self-protein, it proved possible to induce an equally fast and even a stronger autoantibody response against TNFα despite the fact that the inserted T cell epitope used was not restricted to the MHC molecules of the immunized mice, vide Example 3 below. The reason for this observation is not clear but may be due to the appearance of new MHC binding segments in the mutagenized area in the self-protein. However, the experiment shown in example 5 demonstrates that this is probably not the case, since synthetic peptides representing overlapping regions of the implanted ovalbumin T cell epitope in ubiquitin did not bind strongly to any of the MHC class II molecules of the H-2^k mice in which this recombinant molecule was highly immunogenic.

Most of the potential MHC class II binding segments of a protein are normally cryptic and will not be presented to the host T cells by the antigen presenting cells (S. Mouritsen et al, Scand. J. Immunol. 34, 421, 1991). The observed lacking correspondence between the MHC restriction of the inserted T cell epitope and the restriction of the antibody response could perhaps be due to a general disturbance of the intra-molecular competition of binding to MHC molecules by different self-protein segments. According to the method of the invention non-tolerized cryptic self-protein segments may be presented to the T cells leading to breaking of the T cell as well as the B cell autotolerance towards the protein. In accordance with the invention and illustrated in all the examples described below, a fragment of the self-protein was substituted with a foreign T cell epitope. This deletion followed by a substitution with an other protein fragment minimally obscure the tertiary structure of the self-proteins, but may also contribute strongly to the disturbance of said intramolecular competition of the MHC class II binding self-segments. This concept is therefore clearly different from the above-mentioned prior art mechanisms and methods. Independently of the operating mechanism of action by the method according to the invention, it is more technically advantageous compared to the known methods for breaking the B cell autotolerance, since it is possible to induce antibodies in a broad population of MHC molecules by insertion of a minimal number of different foreign T cell epitopes.

The present invention thus is based on the surprising fact that injection of recombinant self-proteins, which have been appropriately modulated by deletion of one or more peptide fragments and simultaneous insertion of a corresponding number of foreign T cell epitopes, so as to produce a self-protein analog with an essentially preserved tertiary structure, induces a profound autoantibody response against the unmodified self-proteins. Surprisingly the MHC-restriction of the auto-antibody response induced was not necessarily confined to that of the inserted T cell epitope. By inducing minimal tertiary structural changes in the highly conserved self-protein ubiquitin, as well as in TNFα, foreign T cell epitopes having a length of 12-15 amino acids were inserted using genetic engineering methods. These recombinant self-protein analogs were purified, emulsified in adjuvant and injected into mice. Within only one week an autoantibody response against ubiquitin could be detected in serum from these mice. Non-modified, recombinant ubiquitin treated and injected in the same way was not able to induce a response.

By using this principle for developing vaccines against undesirable proteins, the risk of inducing allergic side-effect is reduced, and toxic self-proteins such as TNFα can simultaneously be detoxified by removing or mutating biologically active protein segments. The epitope-shielding effect described above is not a problem, and autoantibodies against ubiquitin were induced much faster as compared to the known technique, in which the self-protein is conjugated to a carrier protein or peptide. Importantly, by this method it furthermore seems possible to temporarily break the autotolerance of the T cells as well as that of the B cells of the individual, and such recombinant proteins will be self-immunogenic in a large population expressing many different MHC class II molecules.

The vaccine according to the invention consists of one or more self-protein analogs modulated as described above and formulated with suitable adjuvants, such as calcium phosphate, saponin, quil A or biodegradable polymers. The modulated self-protein analogs may be prepared as fusion proteins with suitable, immunologically active cytokines, such as GM-CSF or interleukin 2.

The autovaccine may i.a. be a vaccine against TNFα or γ-interferon for the treatment of patients with cachexia, e.g. cancer patients, or a vaccine against IgE for the treatment of patients with allergy. Furthermore, it may be a vaccine against TNFα, TNFβ or interleukin 1 for the treatment of patients with chronic inflammatory diseases.

The invention is illustrated in the following examples:

EXAMPLE 1

Substitution of foreign T cell epitopes into ubiquitin

An overview of this procedure is shown in fig. 1 using the T cell epitope MP7 as example. The gene sequences representing MP7 (MP7.1-C and MP7.1-NC) were synthesized as two complementary oligonucleotides designed with appropriate restriction enzyme cloning sites. The amino acid sequence of MP7 is PELFEALQKLFKHAY, Mouritsen et al, Scand. J. Immunol. 30, 723-730, 1989. The oligonucleotides were synthesized using conventional, automatic solid phase oligonucleotide synthesis and purified using agarose gel electrophoresis and low melting agarose. The desired bands were cut out from the gels, and known quantities of oligonucleotides were mixed, heated to 5C below their theoretical melting point (usually to approximately 65C) for 1-2 hours, and slowly cooled to 37C. At this temperature the hybridized oligonucleotides were ligated to the vector fragments containing part of the ubiquitin gene. The subsequent analysis of positive clones using restriction fragment analysis and DNA sequencing was done by conventional methods ("Molecular Cloning", Eds.: T. Maniatis et al. 2 ed. CSH Laboratory Press, 1989).

EXAMPLE 2

Induction of autoantibodies against ubiquitin by vaccination with modified ubiquitin analogs

Genes containing sequences encoding the foreign T cell epitopes OVA (325-336) from ovalbumin (OVA) and HEL (50-61) from hen eggwhite lysozyme (HEL), respectively, were expressed in E. coli strain, AR58 under control of the heat sensitive S repressor regulated promotor. Expression of the recombinant ubiquitin proteins were verified using a polyclonal anti-ubiquitin antibody and Western-blotting ("Antibodies", Eds.: D. Harlow et al., CSH Laboratory Press, 1988). The recombinant proteins were purified using conventional methods (Maniatis et al., supra).

Mice were injected i.p. with 100 μg of ubiquitin or its analogs in phosphate buffered saline (PBS) emulsified in Freunds Complete adjuvant. Booster injections of the same amount of antigen emulsified 1:1 in Freunds Incomplete adjuvant were performed i.p. at days 14 and 28. Five Balb/c mice in each group were examined and blood samples were examined for the presence of anti-ubiquitin antibodies on day 7, 14, 21, 28, 35, and 42 using conventional ELISA methodology.

The results exemplified by the antibody response against two different ubiquitin analogs containing the T cell epitopes OVA(325-336) and HEL(50-61), respectively, are shown in fig. 2. The amino acid sequence of the inserted OVA(325-336) epitope is: QAVHAAHAEINE and the amino acid sequence of the HEL(50-61) epitope is STDYGILQINSR.

A clear antibody response against native ubiquitin could be detected within only one week from the first injection of antigen reaching a maximum within 2 weeks. Antiubiquitin antibodies produced in rabbits by covalently conjugating ubiquitin to bovine immunoglobulin reached maximum values after a much longer immunization period (data not shown).

EXAMPLE 3

Induction of autoantibodies against tumor necrosis factors (TNF) by vaccination with appropriately modified TNF analogs

The gene coding for the structural part of the native murine TNFα protein (MR101) was obtained by Polymerase Chain Reaction (PCR) cloning of the DNA. In the MR103 TNFα analog the ovalbumin (OVA) H-2^d restricted T cell epitope sequence 325-334 (QAVHAAHAET) replaces the amino acids 26-35 in the cloned TNFα sequence, a substitution of an amphiphatic α-helix. Substitutions in this region of the TNFα detoxifies the recombinant protein (X. Van Ostade et al., Nature 361, 266-269, 1993). In the MR105 analog the H-2^k restricted T cell epitope from hen eggwhite lysozyme (HEL), amino acid sequence 81-96 (SALLSSDITASVNCAK) replaces the amino acids 5-20 in the cloned TNFα sequence. In the MR106 TNFα mutant the same epitope, amino acid sequence 81-95 (SALLSSDITASVNCA) replaces the amino acids 126-140 in the cloned TNFα sequence. The genetic constructions are shown in Fig. 3. Different techniques compared to the technique described in example 1 were used for exchanging parts of the TNFα gene with DNA coding for T cell epitopes. The MR105 and 106 constructs were made by introducing the mutant sequence by PCR recloning a part of the TNFα gene flanking the intended site for introducing the T cell epitope. The mutant oligonucleotide primer contained both a DNA sequence homologous to the TNFα DNA sequence as well as a DNA sequence encoding the T cell epitope. The PCR recloned part of the TNFα gene was subsequently cut with appropriate restriction enzymes and cloned into the MR101 gene. The MR103 construction was made by a modification of the "splicing by overlap extension" PCR technique (R. M. Horton et al., Gene 77, 61, 1989). Here two PCR products are produced, each covering a part of the TNFα gene, and additionally each PCR product contains half of the T cell epitope sequence. The complete mutant TNFα gene was subsequently made by combining the two PCR products in a second PCR. Finally, the complete genetic constructions were inserted into protein expression vectors. Subsequently, all genetic constructions were analyzed by restriction fragment analysis and DNA sequencing using conventional methods ("Molecular Cloning", Eds,: T. Maniatis et al. 2.ed. CSH Laboratory Press, 1989). The recombinant proteins were expressed in E.coli and purified by conventional protein purification methods.

Groups of BALB/c (MHC haplotype H-2^d) and C3H (MHC haplotype H-2^k) mice, respectively, were immunized subcutaneously with 100 Tg of semi-purified MR103 and MR106 emulsified in Freunds' complete adjuvant. Every second week the immunizations were repeated using incomplete Freunds' adjuvant. All mice developed an early and strong antibody response against biologically active MR101. This was measured by a direct ELISA method using passively adsorbed 100% pure MR101 (Fig. 4). Control mice immunized with MR101 and PBS, respectively, showed no antibody reactivity towards MR101.

Strikingly, the antibody response towards MR101 was not MHC restricted corresponding to the implanted T cell epitopes, since both mice strains of different MHC haplotypes responded well to MR103 and MR106 containing differently restricted T-cell epitopes (Fig. 4). Taken together these results illustrate (a) the ability of the self-protein analogs produced by the method according to the invention to induce autoantibodies towards a secreted autoprotein and (b) the improved efficiency of the herein described method with regard to inducing a response in a broader MHC population than predicted by the MHC binding ability of the inserted T cell epitopes. The immune response against MR101 induced by the recombinant self-protein analogs MR103 and MR106 was much stronger and more high-titered compared to the immune response induced by aldehyde conjugated MR101 (see Example 4).

EXAMPLE 4

Induction of autoantibodies against TNFα by self-protein analogs produced by the method according to the invention compared to unmodified self-protein conjugated to E. coli proteins.

The induction of autoantibodies against TNFα by the method of the present invention has been directly compared to the autoantibody response induced when using a conjugate of TNFα and E. coli proteins, which must contain small single T cell epitope peptides as well as larger foreign carrier proteins.

Semi-purified recombinant murine TNFα (MR101) was conjugated to E. coli proteins in PBS, pH 7.4, using 0.5% formaldehyde. Conjugation of the proteins was confirmed by SDS-PAGE. These conjugates were subsequently used for immunization of C3H mice. Another group of C3H mice was vaccinated with semi-purified non-conjugated self-protein analog MR105. About 100 μg of recombinant TNFα analog and conjugate were emulsified 1:1 in Freunds' complete adjuvant and injected subcutaneously in each group of mice. MR105 is biologically inactive as judged by the L929 bioassay for TNFα. In subsequent immunizations every second week incomplete Freunds' adjuvant was used. Both groups eventually developed autoantibodies against highly purified biologically active MR101 as determined by ELISA, but the immune response against the non-conjugated analog MR105 produced by the method of the invention was induced earlier and was of a higher titer (Fig. 5).

EXAMPLE 5

The possible MHC class II binding of peptides representing overlapping sequences of self-protein as well as of the ovalbumin T cell epitope inserted in ubiquitin.

Peptide-MHC complexes were obtained by incubating ^{125}I-labeled peptide (10-100 nM) with affinity purified MHC class II molecules (2-10 μM) at room temperature for 3 days (S. Mouritsen, J. Immunol. 148, 1438-1444, 1992). The following peptides were used as radiolabeled markers of binding: Hb(64-76)Y which binds strongly to the E^k molecule and HEL(46-61)Y which binds strongly to the A^k molecule. These complexes were co-incubated with large amounts of cold (non-radiolabeled) peptide (> 550 Tm) which is sufficient to inhibit totally all immunologically relevant MHC class II binding. Either the same peptides were used, or three different overlapping peptides were used, said peptides representing the flanking regions as well as the entire OVA(325-336) T cell epitope which was substituted into ubiquitin (see Example 2). The three peptides were: TITLEVEPSQAVHAA (U(12-26)), PSQAVHAAHAEINEKE (U(19-34)) and HAEINEKEGIPPDQQ (U(27-41)). The reaction buffer contained 8 mM citrate, 17 mM phosphate, and 0.05% NP-40 (pH 5) and peptide-MHC class II complexes were separated (in duplicate) from free peptide by gel filtration using G25 spun columns. Both the radioactivities of the excluded "void" volume and of the included volume were measured by gamma spectrometry. The competitive inhibition of maximal binding (in percent) by addition of cold peptide was calculated. The results are shown in Table I.

TABLE I

Peptide/MHC	Hb(64-76)	HEL(46-61)	U(12-26)	U(19-34)	U(27-41)
A^k	28.6	97.4	35.3	44.6	7.8
E^k	92.6	0.0	45.6	12.2	0.0

It can be seen that total inhibition of the binding of the radiolabeled peptides Hb(64-76)Y and HEL(46-61)Y to E^k and A^k respectively could only be achieved using cold versions of the same peptides. Although some inhibition of binding was seen by U(12-26) and U(19-34) using these extreme amounts of cold peptide, it is likely that the affinity of these peptides to the H-2^k MHC class II molecules is very low. Therefore this seems not to be sufficient to explain the strong immunogenicity in the H-2^k mouse strain of the ubiquitin analog containing the ovalbumin T cell epitope. More likely, other and previously non-tolerized self-epitopes are presented to the T cell in these animals.

EXAMPLE 6

Treatment of diabetes or inflammatory disease by vaccination with appropriately modified TNFα analogs

Genes coding for TNFα are modified by insertion of appropriate gene segments coding for T cell epitopes derived from e.g. tetanus toxin or influenza hemagglutinin. Such genes are expressed in appropriate expression vectors in e.g. E. coli or insect cells. The recombinant TNFα proteins were purified using conventional methods ("Molecular Cloning", Eds.: T. Maniatis et al. 2. ed. CSH Laboratory Press, 1989).

Optionally such recombinant proteins can be coupled to immunologically active cytokines such a GMCSF or interleukin 2.

The recombinant proteins can be formulated with appropriate adjuvants and administered as an anti-TNFα vaccine to patients suffering from diseases where TNFα is important for the pathogenesis. The induced anti-TNFα antibodies will thereby affect the diseases.

One example of said diseases is the chronic inflammatory diseases such as e.g. rheumatoid arthritis where TNFα is believed to play an important role (reviewed in: F.M. Brennan et al., Br. J. Rheumatol. 31, 293-298, 1992). TNFα is also believed to play an important role in the cachec tic conditions seen in cancer and in chronic infectious diseases such as AIDS (reviewed in M. Odeh. J. Intern. Med. 228, 549-556, 1990). It is also known that TNF participates in septic shock (reviewed in: B.P. Giroir, Crit. Care. Med., 21, 780-789, 1993). Furthermore, it has been shown that TNFα may play a pathogenetic role in the development of type II diabetes mellitus (CH Lang et al., Endocrinology 130, 43-52, 1992).

LEGENDS TO FIGURES

Fig. 1. Schematic overview of the cloning strategy used in the construction of a ubiquitin gene with an implanted

foreign T cell epitope (MP7). Restriction enzyme digestions, hybridization and ligation procedures are indicated with arrows. Fragment sizes are shown in parentheses.

Fig. 2. Reactivity toward immobilized bovine ubiquitin in sera from mice immunized with recombinant ubiquitin and analogs containing the implanted T cell epitopes OVA(325-336) and HEL (50-61), respectively. Fig. 2a) sera from Balb/c mice immunized with recombinant ubiquitin containing OVA(325-336). Fig. 2b) sera from Balb/c mice immunized with recombinant ubiquitin containing the T cell epitope HEL(50-61). Fig. 2c) sera from Balb/c mice immunized with recombinant non-modified ubiquitin. Sera (diluted 1:100) were tested in a standard ELISA assay using non-modified bovine ubiquitin immobilized on the solid phase.

Fig. 3. Schematic overview of the cloning strategy used in the construction of the recombinant TNFα mutants. PCR products and restriction enzyme digestions are indicated.

Fig. 4. Induction of TNFα autoantibodies by vaccination of Balb/c or C3H mice with semipurified MR103 and MR106. The antibody titers were measured by ELISA and expressed as arbitrary units (AU) referring to a strong standard anti-serum from one mouse. The plotted values represent a mean titer for 5 animals. Freunds complete adjuvant was used as adjuvant for the first immunization. All subsequent immunizations at 14 days intervals were done with Freunds incomplete adjuvant. Mice immunized in parallel with native MR101 in PBS did not develop detectable TNFα autoantibodies (data not shown). Non-detectable antibody titers were assigned the titer value 1.

Fig. 5. Anti TNFα autoantibodies induced by vaccination with non-conjugated MR105 and MR101 conjugated to E. coli proteins, respectively. C3H mice and Balb/c mice were immunized with both preparations. The immunizations, measurements and calculations of mean antibody titers were done as described in example 4.

Claims

1. A method for the modification of self-proteins so as to induce antibody response against the unmodified self-proteins following administration of said modified self-proteins to the host, CHARACTERIZED by providing a self-protein analog by molecular biological means by substitution of one or more peptide fragments of the self-protein by a corresponding number of peptides known to contain immunodominant foreign T cell epitopes, said substitution being carried out so as to essentially preserve the overall tertiary structure of the original self-protein.

2. A method according to claim 1, wherein said immunodominant foreign T-cell epitopes are inserted so as to preserve flanking regions from the original self-protein on both sides of the T-cell epitope.

3. A method according to any of claims 1 or 2, wherein the immunodominant T cell epitope(s) originate(s) from tetanus toxoid or diphtheria toxoid.

4. An autovaccine against undesirable self-proteins in humans or animals, CHARACTERIZED in that it comprises one or more self-protein analogs modified according to any of claims 1 - 3 and formulated with pharmaceutically acceptable adjuvants, such as calcium phosphate, saponin, quil A and biodegradable polymers.

5. An autovaccine according to claim 4, CHARACTERIZED in that the self-protein analog is present in the form of a fusion protein with suitable, immunologically active cytokines, such as GM-CSF or interleukin 2.

6. An autovaccine according to claim 4, CHARACTERIZED in that it is a vaccine against TNFα or γ-interferon for the treatment of patients susceptible to cachexia, e.g. cancer patients.

7. An autovaccine according to claim 4, CHARACTERIZED in that it is a vaccine against IgE for the treatment of patients with allergy.

8. An autovaccine according to claim 4, CHARACTERIZED in that it is a vaccine against TNFα, TNFβ or interleukin 1 for the treatment of patients with chronic inflammatory diseases.

9. An autovaccine according to claim 8, CHARACTERIZED in that it is a vaccine for treatment of patients with rheumatoid arthritis or an inflammatory bowel disease.

10. An autovaccine according to claim 4 or 5, CHARACTERIZED in that it is a vaccine against TNFα for the treatment of diabetes mellitus.

Patentansprüche

1. Verfahren zur Modifizierung von Eigenproteinen, so daß diese eine Antikörperantwort gegen nicht-modifizierte Eigenproteine nach Verabreichung der modifizierten Eigenproteine an den Wirt induzieren, **gekennzeichnet** durch Bereitstellung eines Eigenproteinanalogs mittels Molekularbiologie durch Substitution von einem oder mehrerer Peptidfragmente des Eigenproteins durch eine entsprechende Anzahl von Peptiden, von denen man weiß, daß sie immundominante Fremd-T-Zell-Epitope enthalten, wobei die Substitution so durchgeführt wird, daß im wesentlichen die Gesamt-Tertiärstruktur des ursprünglichen Eigenproteins erhalten bleibt.

2. Verfahren nach Anspruch 1, worin die immundominanten Fremd-T-Zell-Epitope so eingefügt werden, daß die flankierenden Regionen aus dem ursprünglichen Eigenprotein an beiden Seiten des T-Zell-Epitops erhalten bleiben.

3. Verfahren nach einem der Ansprüche 1 oder 2, worin das (die) immundominante(n) T-Zell-Epitop(e) aus Tetanustoxoid oder Diphtherietoxoid stammt (en).

4. Autoimpfstoff gegen unerwünschte Eigenproteine in Menschen oder Tieren, **gekennzeichnet** darin, daß es ein oder mehrere Eigenproteine, modifiziert nach einem der Ansprüche 1 bis 3, enthält und mit pharmazeutische verträglichen Adjuvanzien, wie Kalziumphosphat, Saponin, Quil A und bioabbaubaren Polymeren formuliert ist.

5. Autoimpfstoff nach Anspruch 4, dadurch **gekennzeichnet,** daß das Eigenproteinanalog in Form eines Fusionsproteins mit geeigneten, immunologisch aktiven Cytokinen, wie GM-CSF oder Interleukin 2, vorliegt.

6. Autoimpfstoff nach Anspruch 4, dadurch **gekennzeichnet,** daß es ein Impfstoff gegen TNFα oder γ-Interferon zur Behandlung von Patienten ist, die für Cachexie anfällig sind, z. B. Krebspatienten.

7. Autoimpfstoff nach Anspruch 4, dadurch **gekennzeichnet,** daß es ein Impfstoff gegen IgE zur Behandlung von Patienten mit Allergie ist.

8. Autoimpfstoff nach Anspruch 4, dadurch **gekennzeichnet,** daß es ein Impfstoff gegen TNFα, TNFβ oder Interleukin 1 zur Behandlung von Patienten mit chronischen Entzündungskrankheiten ist.

9. Autoimpfstoff nach Anspruch 8, dadurch **gekennzeichnet,** daß es ein Impfstoff zur Behandlung von Patienten mit rheumatoider Arthritis oder entzündlicher Darmerkrankung ist.

10. Autoimpfstoff nach Anspruch 4 oder 5, dadurch **gekennzeichnet,** daß es ein Impfstoff gegen TNFα zur Behandlung von Diabetes mellitus ist.

Revendications

1. Procédé de modification de protéines du soi de manière à induire une réponse anticorps contre les protéines du soi non modifiées après administration chez l'hôte desdites protéines du soi modifiées, caractérisé en ce que l'on fournit un analogue de la protéine du soi par des moyens biologiques par substitution d'un ou plusieurs fragments peptidiques de la protéine du soi par un nombre correspondant de peptides connus pour contenir des épitopes immunodominants de cellules T exogènes, ladite substitution étant effectuée de manière à essentiellement préserver la structure tertiaire globale de la protéine du soi d'origine.

2. Procédé selon la revendication 1, dans lequel lesdits épitopes immunodominants de cellules T exogènes sont insérés de manière à préserver les régions bordantes de la protéine du soi d'origine sur les deux côtés de l'épitope de la cellule T.

3. Procédé selon la revendication 1 ou 2, dans lequel le (ou les) épitope(s) immunodominants de cellules T proviennent de l'anatoxine tétanique ou de l'anatoxine diphtérique.

4. Autovaccin contre les protéines du soi indésirables chez l'homme ou l'animal, caractérisé en ce qu'il comprend un ou plusieurs analogues modifiés d'une protéine du soi selon l'une quelconque des revendications 1 a 3, et formulés avec des adjuvants pharmaceutiquement acceptables, tels que le phosphate de calcium, saponine, quil A et les polymères biodégradables.

5. Autovaccin selon la revendication 4, caractérisé en ce que l'analogue de la protéine du soi est présente sous la forme d'une protéine de fusion avec des cytokines immunologiquement actives appropriées, tels que GM-CSF ou l'interleukine 2.

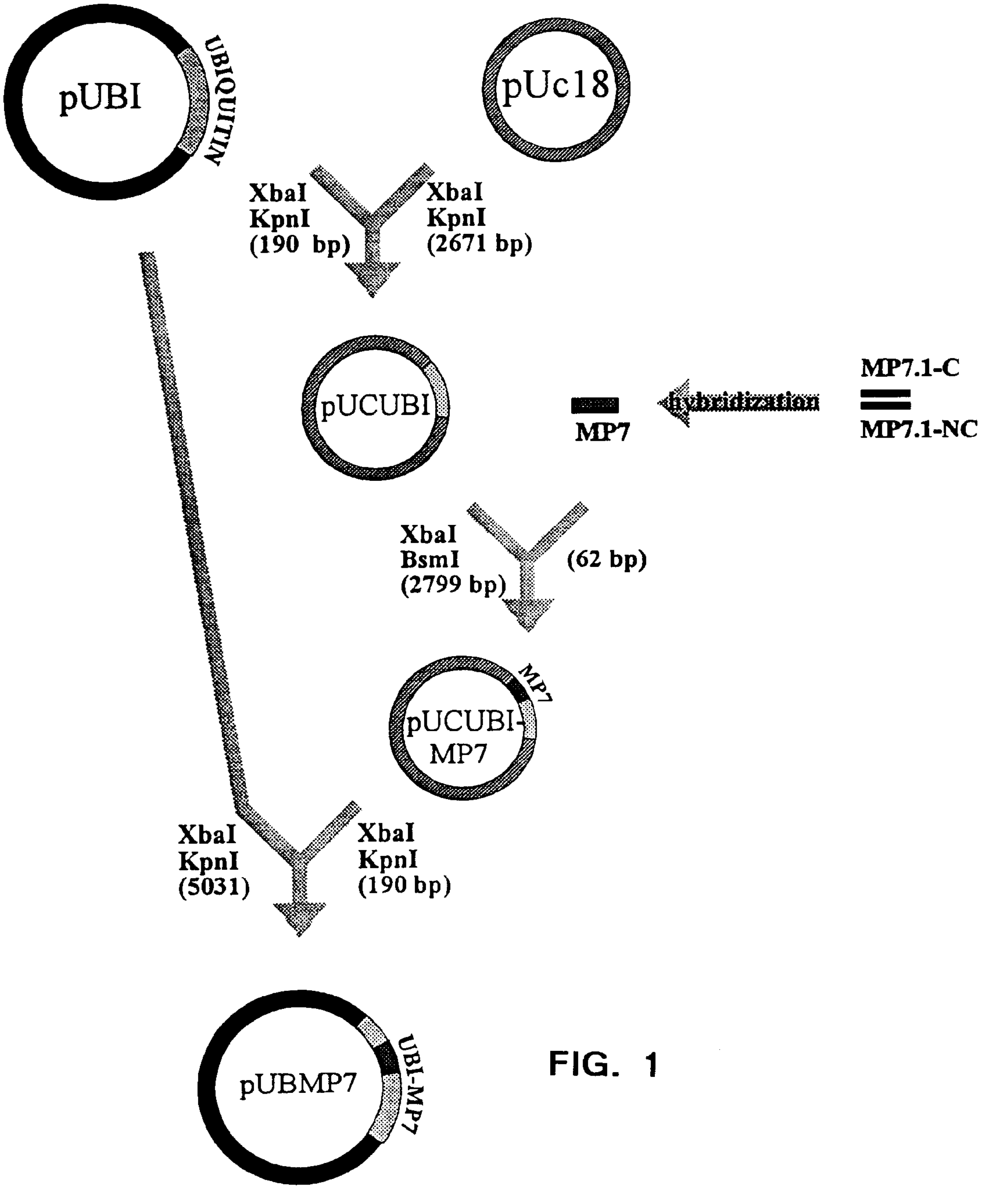
6. Autovaccin selon la revendication 4, caractérisé en ce qu'il représente un vaccin contre TNFα ou l'interféron-γ pour le traitement de patients atteints de cachexie, par exemple des patient atteints d'un cancer.

7. Autovaccin selon la revendication 4, caractérisé en ce qu'il représente un vaccin contre les IgE pour le traitement des patients allergiques.

8. Autovaccin selon la revendication 4, caractérisé en ce qu'il représente un vaccin contre TNFα, TNFβ, ou l'interleukine 1, pour le traitement de patients atteints de maladies inflammatoires chroniques.

9. Autovaccin selon la revendication 8, caractérisé en ce qu'il représente un vaccin pour le traitement de patients atteints de rhumatisme articulaire ou de maladie inflammatoire intestinale.

10. Autovaccin selon la revendication 4 ou 5, caractérisé en ce qu'il représente un vaccin contre TNFα pour le traitement des diabètes sucrés.


pUBI
UBIQUITIN
pUc18
XbaI
KpnI
(190 bp)
XbaI
KpnI
(2671 bp)
pUCUBI
MP7
hybridization
MP7.1-C
MP7.1-NC
XbaI
BsmI
(2799 bp)
(62 bp)
pUCUBI-MP7
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XbaI
KpnI
(5031)
XbaI
KpnI
(190 bp)
pUBMP7
UBI-MP7

FIG. 1

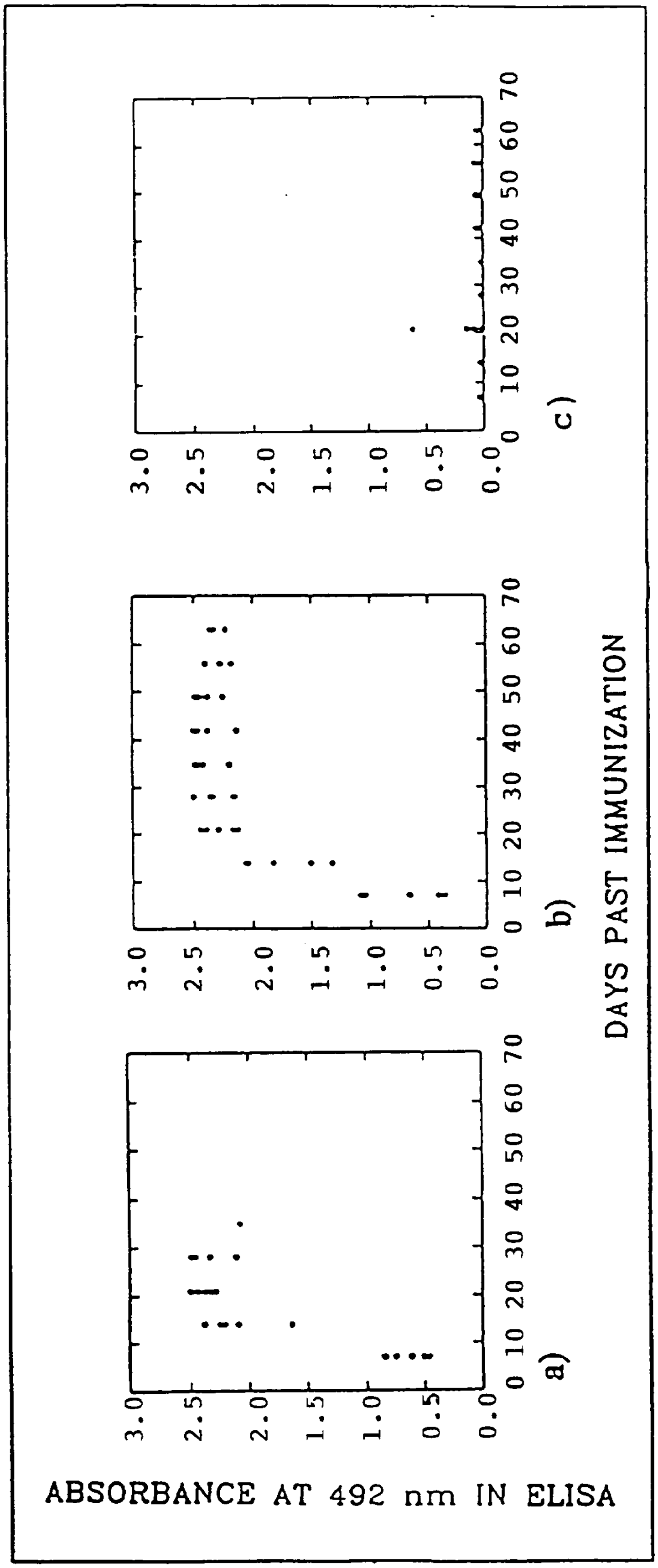
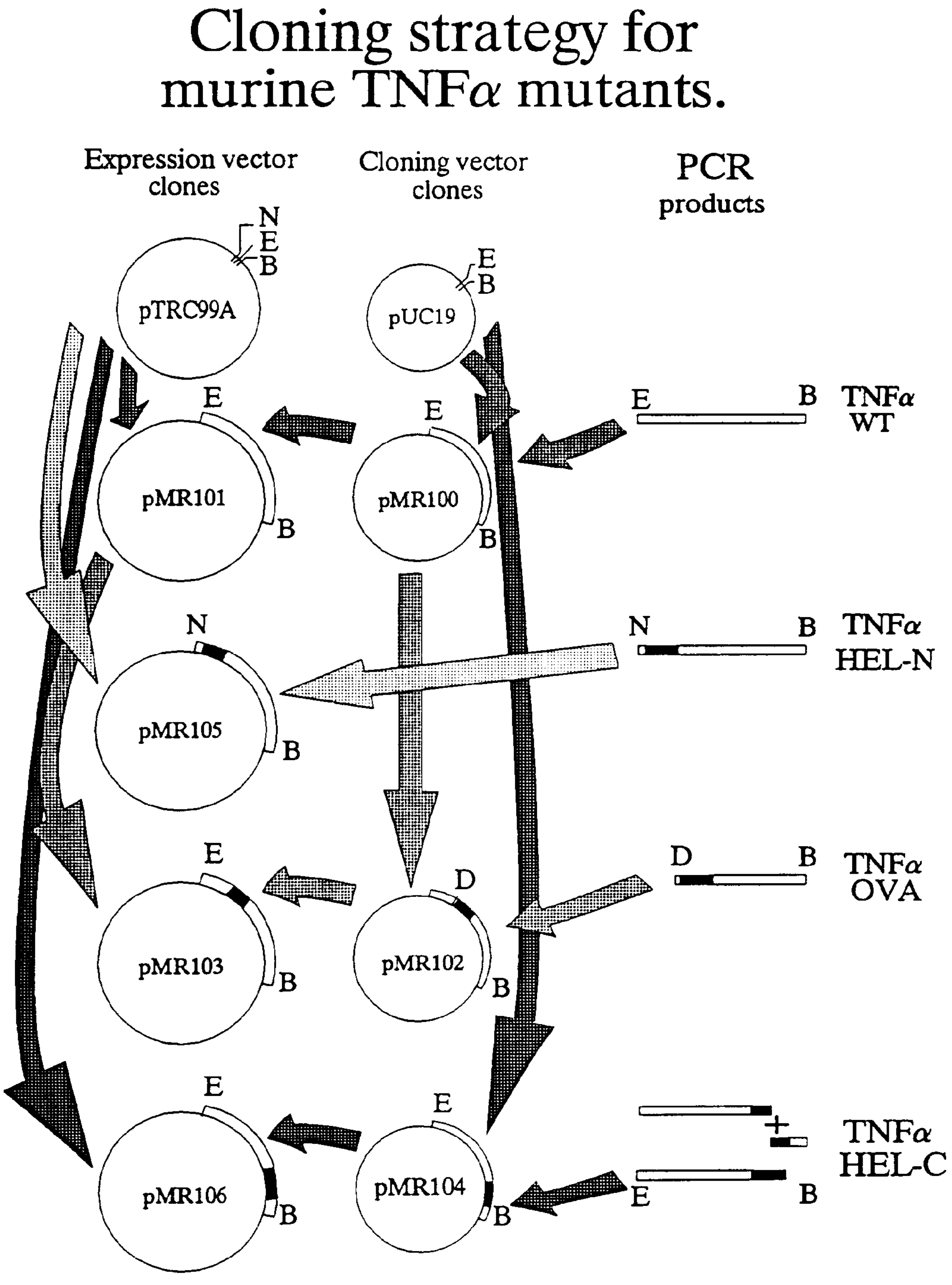

ABSORBANCE AT 492 nm IN ELISA
DAYS PAST IMMUNIZATION
a)
b)
c)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70

FIG. 2



Cloning strategy for murine TNFα mutants.

Restriction enzyme symbols: E: EcoRI, B:BamHI, N: NcoI, D: DraIII.

FIG. 3

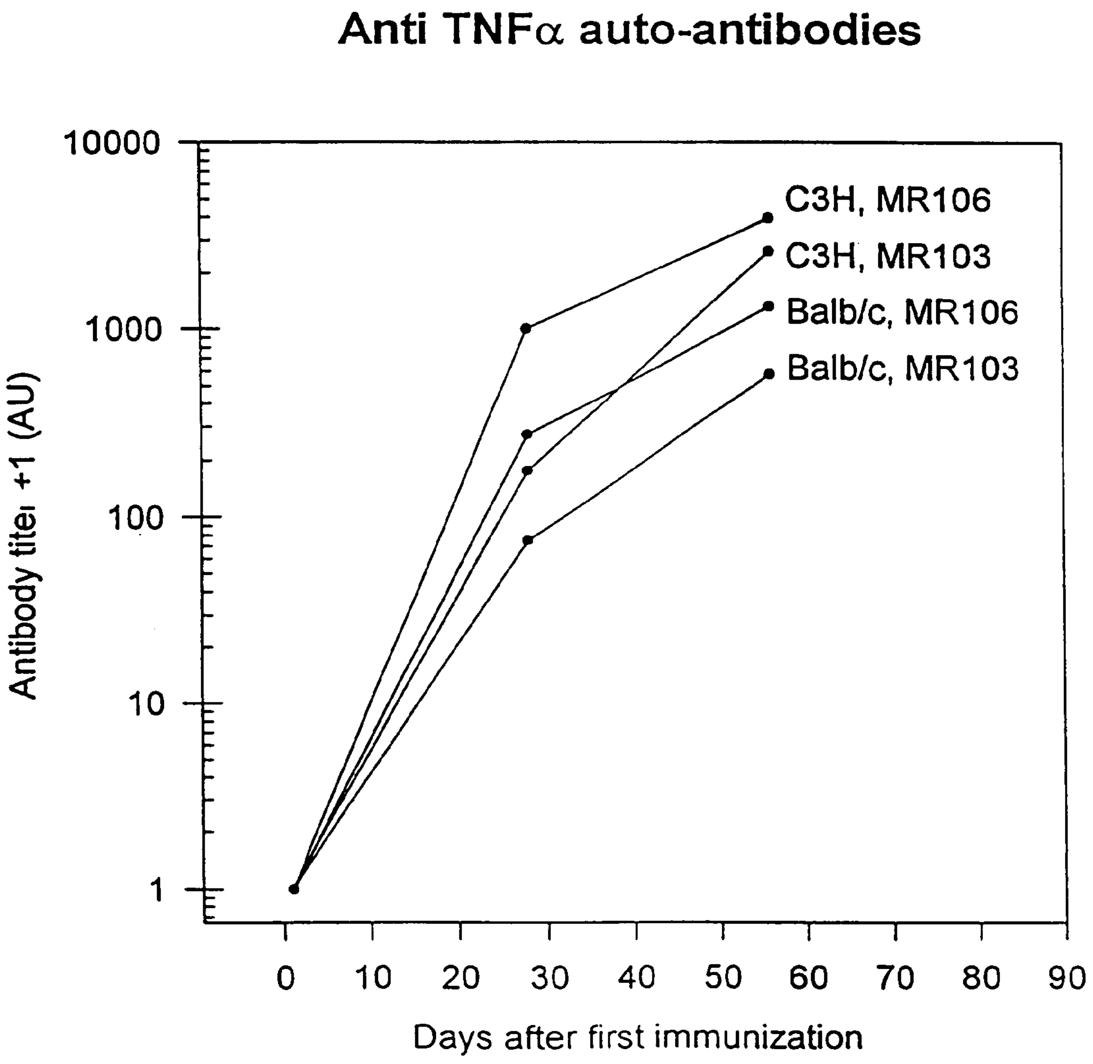

Anti TNFα auto-antibodies
10000
1000
100
10
1
Antibody titei +1 (AU)
0
10
20
30
40
50
60
70
80
90
Days after first immunization
C3H, MR106
C3H, MR103
Balb/c, MR106
Balb/c, MR103

FIG. 4

Anti-TNFα autoantibodies

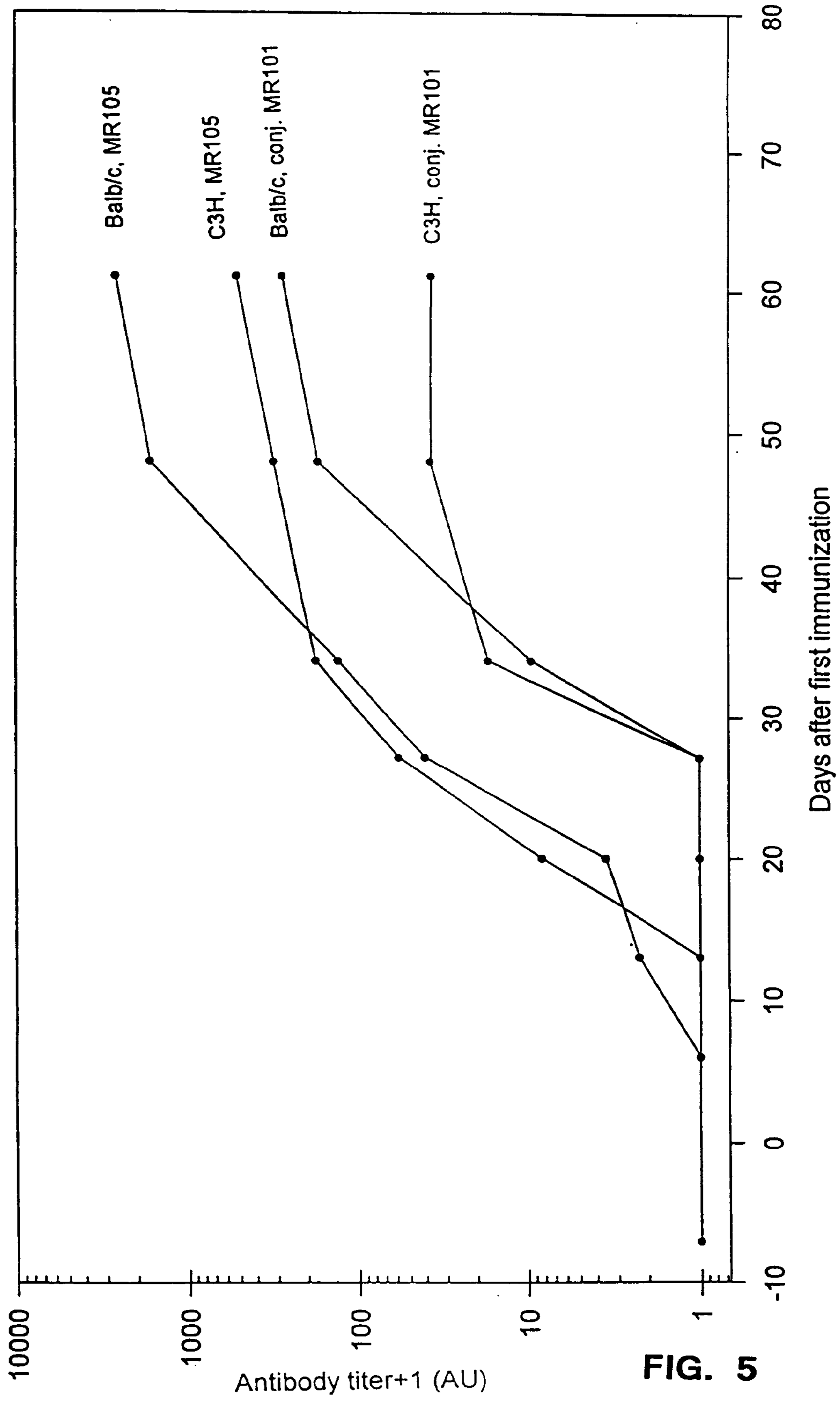


FIG. 5

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(54) Title: VACCINE-MEDIATED TREATMENT OF NEUROLOGICAL DISORDERS

(57) Abstract: A method of treating or preventing development of a neurological disorder has been developed wherein a subject with the disorder, or at risk of developing a disorder, is vaccinated against a brain protein or antigen. Alternatively, the antibodies can be directly administered to the individual in need of treatment thereof. Animal studies demonstrate potent efficacy in the treatment of epilepsy, stroke and cognition in animal models vaccinated against the NMDA receptor.

VACCINE-MEDIATED TREATMENT OF NEUROLOGICAL DISORDERS

Background of the Invention

The present invention is generally in the field of methods and compositions of treatment of neurological disorders, such as epilepsy and stroke, and neuroendocrine disorders, such as obesity. The present invention is also in the field of methods and compositions for modulating gene expression, such as gene expression of the N-methyl-D-aspartate (NMDA) receptor and the transcription factor, Krox-24.

Methods for treating a variety of neurological disorders have focused on the use of pharmaceutical agents which interact with neurological receptors such as the NMDA receptor, neurotransmitter transporters, such as the serotonin or dopamine transporters, various ion channels, or compounds which act to supplement or replace a neurotransmitter such as dopamine. Numerous treatments have been proposed for treatment of disorders such as Alzheimer's, Parkinson's and damage due to stroke, all without lasting success.

Due to NMDA receptors central involvement in the cascade leading to neuronal death following a variety of cerebral insults, pharmacological NMDA receptor antagonists have been evaluated for potential clinical utility. These drugs have shown to be effective in many experimental animal models and some of the compounds have moved into clinical trials (Schehr (1996) *Nat. Biotechnol.* 14:1549-1554). The initial enthusiasm for this approach has, however, waned as the therapeutic ratio for most NMDA antagonists is poor since at clinically effective doses they have been associated with significant adverse effects thereby limiting their utility (Schehr (1996) *Nat. Biotechnol.* 14:1549-1554).

An alternative approach to modify the function of brain proteins has been the use of antisense oligonucleotides or RNA antisense expressing vectors as well as local application of antibodies targeting the specific protein. Some of these approaches have been used to block or translationally suppress NMDA receptor expression and appear effective in a variety of model systems (Wahlestedt *et al.* (1993) *Nature* 363:260-263; Sun and Faden (19950 *Brain Res.* 693:163-168). However, these therapies generally have transient and limited efficacy.

Therefore a need exists to provide an alternative method of treatment for neurological disorders such as epilepsy, stroke, neuropsychiatric and neurodegenerative disorders. A need also exists to target specific antigens within the brain and modify their function.

Summary of the Invention

The invention is based on the surprising discovery that one can immunize a subject with, or at risk of developing, epilepsy, stroke or other neurological disorder, against an antigen, e.g., the NMDA receptor, preferably using a genetic vaccine encoding the NMDA receptor as the immunizing agent, to treat or limit the development of the neurological disorder. Antibodies are produced which are immunoreactive to the antigen, for example, the NMDA receptor and can cross the blood brain barrier, although at very low levels other than during injury or due to a disease process or excessive neuronal activity. When the blood-brain-barrier is compromised, the transfer of the antibody into the brain increases significantly. Alternatively, the antibodies can be administered (passive transfer of immunity) to achieve a similar result. Antibodies that can be administered include humanized antibodies, monoclonal antibodies, polyclonal antibodies, or antibody fragments. The invention also features methods and compositions for treating, or preventing the onset of neuroendocrine disorders, such as, obesity by modifying target molecules involved in the regulation of such disorders. The invention also provides methods of modifying target genes or target proteins and processed involving such targets.

Accordingly, in one aspect, the invention features method for treating a neurological disorder in a subject comprising:

administering a vaccine comprising a therapeutically effective amount of an antigen, wherein the antigen elicits the production of antibodies in the circulatory system of the subject, or a composition comprising a therapeutically effective amount of an isolated antibody, or an antibody portion, wherein the antibodies bind to, and modify the function of a target protein in the central nervous system, to thereby ameliorate or prevent the onset of a neurological disorder in the subject.

In one embodiment, the antibodies pass across the blood-brain barrier into the central nervous system facilitated by injury, disease or excessive neuronal activity.

In one embodiment, the disorder is selected from the group consisting of epilepsy, stroke, Alzheimer's, Parkinson's, dementia, Huntington's disease, amyloid lateral sclerosis and depression. In a preferred embodiment, the neurological disorder is stoke. In another preferred embodiment, the neurological disorder is epilepsy.

In one embodiment, the vaccine comprises an antigen selected from the group of neurotransmitters, neuroreceptors, transporters, ion channels, signal transduction molecules, enzymes involved in the synthesis or degradation of neurotransmitters, growth factors, transcription factors, and cell surface molecules. In a preferred embodiment, the antigen is an NMDA receptor. In a more preferred embodiment, the antigen is NMDAR1.

In one embodiment, the vaccine is selected from the group consisting of a viral vector vaccine, a DNA vaccine, a peptide vaccine and a crude antigen vaccine, or a combination thereof. In another embodiment, the vaccine is a viral vector vaccine comprising a viral vector selected from the group consisting of an RNA viral vector and a DNA viral vector. The viral vector vaccine comprises a viral vector selected from the group consisting of an adenovirus vector, a herpes virus vector, a parvovirus vector, and a lentivirus vector. In a preferred embodiment, the viral vector is an adeno-associated virus vector.

In one embodiment, the step of administering a composition comprising a therapeutically effective amount of an isolated antibody, or an antibody portion, further comprises administering an antibody, or an antibody portion elicited in a mammal for administration to the subject. In another embodiment, the isolated antibody, or antibody portion is administered directly to the central nervous system.

In one embodiment, the isolated antibody, or an antibody portion is selected from the group consisting of a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a chimeric antibody, a humanized antibody, an Fab fragment, an $F(ab')_2$ fragment and a single chain Fv fragment.

In another embodiment wherein the isolated antibody, or antibody portion is selected from the group consisting of an anti-NMDA antibody, an anti-GluR antibody, an anti-NK-1 antibody, an anti-dopamine transporter antibody and anti-glutamic acid decarboxylase antibody. In a preferred embodiment, the isolated antibody, or an antibody portion is an anti-NMDA antibody. In a more preferred embodiment, the isolated antibody, or an antibody portion is an anti-NMDAR1 antibody. In another preferred embodiment, the isolated antibody, or antibody portion is an anti-GluR antibody. In a more preferred embodiment, the isolated antibody is an anti-GluR4 antibody or an anti-GluR6 antibody.

In another aspect, the invention features a method for modifying the function of a target protein in the central nervous system of a subject comprising:

administering a vaccine comprising a therapeutically effective amount of an antigen, wherein the antigen elicits the production of antibodies in the circulatory system of the subject, or a composition comprising a therapeutically effective amount of an isolated antibody, or an antibody portion, wherein the antibodies bind to, and modify the function of a target protein in the central nervous system, to thereby modify the function of the target protein.

In one embodiment, the antibodies pass across the blood-brain barrier into the central nervous system facilitated by injury, disease or excessive neuronal activity.

In one embodiment, the target protein is selected from the group of neurotransmitters, neuroreceptors, transporters, ion channels, signal transduction molecules, enzymes involved in the synthesis or degradation of neurotransmitters, growth factors, transcription factors and cell-surface molecules.

In one embodiment, the vaccine comprises an antigen selected from the group of neurotransmitters, neuroreceptors, transporters, ion channels, signal transduction molecules, enzymes involved in the synthesis or degradation of neurotransmitters, growth factors, transcription factors and cell surface molecules. In a preferred embodiment, the antigen is selected from the group consisting of an NMDA receptor, a GluR receptor, an NPY neuropeptide, galanin, an NK-1 receptor, a dopamine transporter and glutamic acid decarboxylase. In a more preferred embodiment, the antigen is an NMDA receptor. In the most preferred embodiment, the antigen is NMDAR1.

In another aspect, the invention features a method for improving cognition in a subject comprising:

administering a vaccine comprising a therapeutically effective amount of an antigen, wherein the antigen elicits the production of antibodies in the circulatory system of the subject, or a composition comprising a therapeutically effective amount of an isolated antibody, or an antibody portion, wherein the antibodies binds to, and modify the function of a target protein in the central nervous system, to thereby improve cognition of a subject.

In one embodiment, the antibody or antibody portion is an anti-NMDA antibody that binds to the NMDA receptor and upregulates NMDA receptor expression. In another embodiment, the antibody binds to the NMDA receptor and decreases Krox-24 expression.

In another aspect, the invention features a method for treating a subject with a neuroendocrine disorder, or at the risk of developing a neuroendocrine disorder comprising:

administering a vaccine comprising a therapeutically effective amount of an antigen to a subject, wherein the antigen elicits the production of antibodies in the circulatory system of the subject, or a composition comprising a therapeutically effective amount of an isolated antibody, or an antibody portion, wherein the antibodies bind to, and modifies the function of a target protein in the central nervous system, to thereby ameliorate the neuroendocrine disorder, or to prevent the onset of the neuroendocrine disorder in the subject.

In one embodiment, the neuroendocrine disorder is obesity. In one embodiment, the antigen is selected from the group consisting of neuropeptide-Y (NPY), galanin, cocaine-and amphetamine-regulated transcript (CART), orexin, thyrotropin - releasing hormone (TRH), leptan, corticotropin - releasing hormone (CRH) and pro-opiomelanocortin (POMC). In a preferred embodiment, the antigen is neuropeptide Y or galanin.

In one embodiment, the antibody is selected from the group consisting of anti-NPY antibody, anti-galanin antibody, anti-CART antibody, anti-orexin antibody, anti-TRH antibody, anti-leptan antibody, anti-CRH antibody, and anti-POMC antibody. In a preferred embodiment, the antibody is an anti-NPY antibody or an anti-galanin antibody. In one embodiment, the target protein is selected from the group consisting of NPY neuropeptide and galanin.

In another aspect, the invention features a pharmaceutical composition comprising a therapeutically effective amount of an antigen capable of eliciting the production of antibodies in the circulatory system of the subject, or a therapeutically effective amount of an isolated antibody, or an antibody portion, wherein the antibodies bind to, and modify the function of a target protein in the central nervous system.

In yet another aspect, the invention features a genetic vaccine comprising an antigen and a pharmaceutical acceptable carrier. In one embodiment, the genetic vaccine comprises an antigen is selected from the group consisting of neurotransmitters, neuroreceptors, transporters, ion channels, signal transduction molecules, enzymes involved in the synthesis or degradation of neurotransmitters, growth factors and transcription factors. In preferred embodiment, the antigen is an NMDA receptor. In a more preferred embodiment, the antigen is NMDAR1.

Brief Description of the Drawings

Fig. 1A is a plasmid map of the NMDAR1 construct;

Fig. 1B is an agarose gel showing PCR amplification of the CMV promoter from genomic DNA extracted from the intestine of AAVNMDAR1-vaccinated and AAVlac-vaccinated rats;

Figs. 1C is a photograph showing transduction of HEK 293 cells by AAVNMDAR1 virus analyzed by NMDAR1 immunocytochemistry;

Figs. 1D-1F are photographs of intestinal cells four weeks following peroral AAVNMDAR1 administration using double immunofluorescence analysis with propidium iodide to show the lamina propria (lp) and epithelial (ep) cell layers.

NMDAR1 immunohistochemistry showed NMDAR1 protein expression within these two regions;

Fig. 1G is a photograph of intestinal cells showing lack of NMDAR1 protein expression in AAVlac-treated animals at 4 weeks or 5 months (not shown) following peroral AAVNMDAR1 administration;

Fig. 1H is a photograph of intestinal cells 5 months following peroral AAVNMDAR1 administration;

Figs. 2A-2H are photographs showing NMDAR1 protein expression in lamina propria. Double label immunofluorescent staining combined with acridine orange counterstaining was used to visualize nuclei showed colocalization of NMDAR1 protein (Fig. 2B, and Fig. 2D) with antibodies to gut cell markers (Fig. 2A) SIRP and (Fig. 2C) dendritic cells;

Figs. 3A-3C are photographs of immunoblots of sera from AAVlac, AAVNMDAR1 and naïve control rats screened for the presence of β-galactosidase antibodies;

Figs. 3D-3H are epitope map profiles of five different AAVNMDAR1-treated animals (N11, N19, N21, N52, and N64). Specificity was measured as a ratio between the AAVNMDAR1 signal and mean AAVlac signals for each peptide;

Fig. 4A are electroencephalograph (EEG) recordings displaying the kainate induced seizure damage in the hippocampus and the neuroprotective effect on status epilepticus (SE) in AAVNMDAR1 treated animals;

Figs. 4B-4K show high power images of neurons to analyze hippocampal damage using fluorescent TUNEL labelling (Figs. 4B,D,F,H,J) or clusterin immunohistochemistry (Figs. 4C,E,G,I,K) combined with immunohistochemistry with NeuN, a mature neuronal marker;

Fig. 5A is a photograph demonstrating antibody passage across an intact blood-brain barrier by immunoblot analysis of cerebrospinal fluid (CSF) from AAVlac, AAVNMDAR1 and AAVGAD vaccinated rats;

Figs. 5B-D are photographs of the hippocampal region of the brain using anti-rat IgG immunohistochemistry and demonstrating that IgG penetration was significantly enhanced in the hippocampus following kainate treatment in the hilus and CA2-CA3 region (Fig. 5B, arrows). Fig. 5C is a high powered image of the CA3 region in (Fig. 5B) compared to the same region under basal conditions (arrows, Fig. 5D).

Figs. 5E and 5I are images of immunohistochemistry analysis conducted on control hippocampal sections using IgG purified from AAVNMDAR1 rat serum.

Figs. 5F and 5H are images of immunohistochemistry analysis conducted on control hippocampal sections using IgG purified from AAVlac rat serum or naïve rat serum (not shown). Figs. 5E-5G are images of the CA3 region, and Figs. 5H-5J are images of the hilar region, demonstrating only AAVNMDAR1 purified IgG showed a selective immunoreactive staining pattern which was similar to that found with a commercial polyclonal NMDAR1 antibody (Figs. 5G, and 5J), while both naïve and AAVlac IgG (Figs. 5F,and 5H) produced only low level background staining

Figs. 6A-6G are photographs of the cortex and stratium demonstrating the reduced ischemic damage in cortex and stratium of AAVNMDAR1-vaccinated rats following middle cerebral artery occlusion;

Fig. 6H is a bar chart showing the reduction in the total infarct volume of the cortex and stratium of AAVNMDAR1-vaccinated animals ($n=10$) compared to AAVlac-treated animals ($n=8$) or control naïve rats ($n=10$). $^*P<0.01$. Each bar represents the mean+SEM;

Fig. 7A is a graph showing the effect of vaccination of the behaviour of rats in a line crossing test. Data represents the number of line crossings in 5 min intervals over 5 successive days in AAVlac-treated animals (squares-solid line) or AAVNMDAR1-vaccinated animals (diamond-dashed line);

Fig. 7B is a graph showing the effect of vaccination of the behaviour of rats in a circular track mobility test. In the circular track test, the number of completed circuits in successive days for AAVlac-treated animals ($n=6$) and AAVNMDAR1-vaccinated animals ($n=6$);

Figs. 8A-8I and Figs. 8K-8M are confocal images showing intracellular Ca^{2+} imaging of cultured mesencephalic neurons and AAVNMDAR1 IgG inummoreactivity in mesencephalic and rat hippocampal neurons. Fig. 8J is a bar chart showing the ratio of changes in fluorescent intensity relative to basal levels between AAVlac and AAVNMDAR1 IgG-treated cells;

Figs. 9A-9L are autoradiographs demonstrating NMDA receptor upregulation in the hippocampus of AAVNMDAR1-vaccinated animals using the three markers of NMDA upregulation;

Figs. 9A-9B are ^{3}H-MK-801 autoradiographs;

Figs. 9C-9D show NMDAR1 immunohistochemistry with a commercial antibody;

Figs. 9E-9F shows the results of *in situ* hybridization with NMDAR1 oligonucleotide probes;

Figs. 9G-9H shows the results of *in situ* hybridization with NMDAR2a oligonucleotide probes;

Figs. 9I-9J shows the results of *in situ* hybridization with and NMDAR2b oligonucleotide probes showing increased binding, immunoreactivity and mRNA levels in AAVNMDAR1-vaccinated animals (Figs. 9B,D,F,H,J) compared to AAVlac-treated animals (Figs. 9A,C,E,G,I);

Figs. 9K and 9L show hippocampal mRNA levels of the trk B receptor were not significantly different in AAVlac-treated (Fig. 9K) and AAVNMDAR1-vaccinated animals (Fig. 9L).

Figs. 10A-10I are immunohistochemical images demonstrating antibody passage across an intact blood-brain barrier and the reduction of basal levels of Krox-24 protein within the cortex of AAVNMDAR1-vaccinated animals (Fig. 10C) compared to AAVlac-treated (Fig. 10B), or naïve animals (Fig. 10A). Insets show high-powered images of Krox-24 immunoreactivity;

Fig. 10I shows an immunoblot analysis of the cerebrospinal fluid (CSF) sampled from an AAVlac-treated animal (Lane 1) and AAVNMDAR1-vaccinated animal (Lane 2). A 117 kDa protein band corresponding to NMDAR1 receptor subunit was identified. Increased levels of the 117 kDa protein was found 90 min following kainate treatment (Lane 3);

Figs. 11A-11I are immunohistochemical images demonstrating the lack of inflammatory responses in the brain associated with vaccination;

Figs. 12A-12D are the results of behavioral test performed on rats vaccinated with AAVNMDAR1 and the effect on learning and memory;

Fig. 12A demonstrates errors and latencies recorded on the Barnes Circular Maze test. Data represents the number of line crossings in 5 min intervals over 5 successive days in AAVlac or AAVNMDAR1 rats;

Fig. 12B demonstrates the line crossing and circular track mobility test;

Fig. 12C demonstrates the data from the contextual fear conditioning test for AAVlac-treated and AAVNMDAR1-vaccinated animals (*p=0.025);

Fig. 12D demonstrates the data from the Spontaneous Object Recognition test; and

Fig. 13 is a bar chart demonstrating the effect of vaccination with AAVNMDAR1 on nociception. The latency for escape responses for the tail immersion test, and the latency for escape responses or hindpaw licking in the hot plate test for AAVlac-treated (black bars), and AAVNMDAR1-vaccinated (white bars) animals. Each bar represents the mean±SEM for all animals in that group (* p=0.04 for tail immersion and p=0.02 for hot plate tests, Student's t-test).

Detailed Description of the Invention

The practice of the present invention employs, unless otherwise indicated, conventional methods of virology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. (*See, e.g.*, Sambrook, *et al.* Molecular Cloning: A Laboratory Manual (Current Edition); DNA Cloning: A Practical Approach, Vol. I & II (D. Glover, ed.); Oligonucleotide Synthesis (*N. Gait, ed.*, Current Edition); Nucleic Acid Hybridization (*B. Hames & S. Higgins, eds.*, Current Edition); Transcription and Translation (*B. Hames & S. Higgins, eds.*, Current Edition); CRC Handbook of Parvoviruses, Vol. I & II (*P. Tijessen*, ed.); Fundamental Virology, 2nd Edition, Vol. I & II (*B. N. Fields and D. M. Knipe, eds.*))

So that the invention is more clearly understood, the following terms are defined:

The term "neurological disorder" as used herein refers to an impairment or absence of a normal neurological function or presence of an abnormal neurological function in a subject. For example, neurological disorders can be the result of disease, injury, and/or aging. As used herein, neurological disorder also includes neurodegeneration which causes morphological and/or functional abnormality of a neural cell or a population of neural cells. Non-limiting examples of morphological and functional abnormalities include physical deterioration and/or death of neural cells, abnormal growth patterns of neural cells, abnormalities in the physical connection between neural cells, under- or over production of a substance or substances, *e.g.*, a neurotransmitter, by neural cells, failure of neural cells to produce a substance or substances which it normally produces, production of substances, *e.g.*, neurotransmitters, and/or transmission of electrical impulses in abnormal patterns or at abnormal times. Neurodegeneration can occur in any area of the brain of a subject and is seen with many disorders including, for example, head trauma, stroke, ALS, multiple sclerosis, Huntington's disease, Parkinson's disease, and Alzheimer's disease.

The term "neuroendocrine disorder" as used herein refers to an impairment or absence of a normal neuroendocrine function or presence of an abnormal neuroendocrine function in a subject. For example, neuroendocrine disorders can be characterized by the disturbance in the regulation of mood, behavior, control of feeding behavior and production of substances, such as insulin, neuropeptide-Y (NPY), galanin, cocaine-and amphetamine-regulated transcript (CART), orexin, thyrotropin - releasing hormone (TRH), leptan, corticotropin - releasing hormone (CRH) and pro-opiomelanocortin (POMC).

The term "subject" as used herein refers to any living organism in which an immune response is elicited. The term subject includes, but is not limited to, humans, nonhuman primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs, and the like. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered.

The term "mammal" as used herein refers to a living organism capable of eliciting a humoral immune response to an antigen. The term subject includes, but is not limited to, nonhuman primates such as chimpanzees and other apes and monkey species, sheep, pigs, goats, horses, dogs, cats, mice, rats and guinea pigs, and the like. The mammal can be used to generate antibodies, or antibody portions, that can subsequently be used to vaccinate a subject.

The term "antibody" includes an immunoglobulin molecule comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The term "antigen-binding portion" of an antibody or an "antibody portion" includes fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, NMDA receptor). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.* (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); *See e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (*See e.g.*, Holliger *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, *et al.* (1994) *Structure* 2:1121-1123).

Still further, an antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecules, formed by covalent or non-covalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov, *et al.* (1995) *Human Antibodies and Hybridomas* 6:93-101) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov *et al.* (1994) *Mol. Immunol.* 31:1047-1058). Antibody portions, such as Fab and $F(ab')_2$ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques, as described herein. Preferred antigen binding portions are complete domains or pairs of complete domains.

An "isolated antibody" includes an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds NMDA is substantially free of antibodies that specifically bind antigens other than NMDA). An isolated antibody that specifically binds NMDA may bind NMDA molecules from other species. Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

The term "autoantibody" as used herein refers an antibody against self-antigen, *i.e.* an antibody that reacts with an antigen that is the normal component of the body. For example, an antibody produced by the subject against an antigen within the subject. An autoantibody can be elicited as part of the immune response to an antigen. For example, by vaccination of an antigen or a portion of an antigen, that is capable of eliciting an immune response resulting in the production of antibodies against the antigen.

The term "antigen" or "immunogen" is used interchangeably and refers to a substance or a material that is specifically recognized by an antibody and to which an antibody can be generated. The antigen can be a whole molecule or a portion of a molecule that can elicit an immune response. The term antigen is also intended to include a nucleic acid molecule encoding an antigen, or a peptide or polypeptide comprising an antigenic epitope. An antigen or a portion thereof that can elicit an immune response can be determined using standard methods such as epitope mapping. Examples of antigens include, but are not limited to, receptors, transporters, ion channels, neurotransmitters, and the like.

The term "systemic circulatory system" or "systemic circulation" as used herein refers to the art known use of the term. The systemic circulatory system serves to transport blood through the body. The systemic circulatory system can be used to elicit an immune response using an antigen that results in the production of antibodies against the antigen. These antibodies continue to exist and circulate throughout the body.

The term "central nervous system" or "CNS" as used herein refers to the art recognized use of the term. The CNS pertains to the brain, cranial nerves and spinal cord. The CNS also comprises the cerebrospinal fluid, which fills the ventricles of the brain and the central canal of the spinal cord.

The term "modifies" or "modified" are used interchangeably herein and refer to the up-regulation or down-regulation of a target gene or a target protein. The term modifies or modified also refers to the increase, decrease, elevation, or depression of processes or signal transduction cascades involving a target gene or a target protein. A target protein, can be a receptor, for example, an NMDA receptor. Modification to the NMDA receptor may occur when an antibody to the NMDA receptor binds to the NMDA receptor. These modification may directly affect the NMDA receptor, for example modifications that result in an increase in NMDA receptor number. Alternatively, the modifications may occur as an indirect effect of binding to the target protein. For example, binding of the anti-NMDA receptor antibody to the NMDA receptor can also lead to a change in downstream processes involving the NMDA receptor, such as a reduction the expression of the Krox-24 protein. The modifications can therefore be direct modifications of the target protein, or an indirect modification of a process or cascade involving the target protein. Non-limiting examples of modifications includes modifications of morphological and functional processes, under- or over production or expression of a substance or substances, *e.g.*, a neurotransmitter, by neural cells, failure of neural cells to produce a substance or substances which it normally produces, production of substances, *e.g.*, neurotransmitters, and/or transmission of electrical impulses.

The term "genetic vaccine" as used herein refers to a vaccine composition comprising one or more antigen(s), or antigen portions that are capable of eliciting an immune response that results in the production of antibodies in the circulatory system of a subject. In particular, the genetic vaccine comprises an antigen or portion thereof, that produces an antibody, which can bind to, and modify the function of a target gene or a target protein, directly or indirectly. The term genetic vaccine is also intended to include a vaccine composition that comprises one or more substances (*e.g.*, neuropeptides, neurotransmitters, and the like) that can bind to, and modulate the function of a target gene or a target protein directly, or indirectly.

The invention is described in more detail in the following subsections:

I. Genetic Vaccines

The brain is generally considered immunoprivileged, although increasing examples of immunological responses to brain antigens, neuronal expression of Major Histocompatibility Class I genes and neurological autoimmunity have been recognized. As demonstrated by the examples, an adeno-associated virus (AAV) vaccine can generate autoantibodies which target a specific brain protein, the NRI subunit of the N-methyl-D-aspartate receptor. In one embodiment, the genetic vaccine of the invention comprises one or more antigen(s). The antigen is capable of eliciting a humoral response that results in the production of antibodies against the antigen. The antigen, such as NMDAR1, is selected based on the disorder to be treated or prevented. For example, for disorders in which the NMDA receptor plays a major role, such as epilepsy, stroke, brain trauma, depression, dementia, chronic pain, migraine and neurodegenerative disorders including ALS, Huntington's, Parkinson's and Alzheimer's Diseases, the NMDA receptor or a portion thereof is used as the basis for the vaccine. In addition, for Parkinson's Disease, the dopamine transporter is used as the antigen. Other disorders which can be treated include Alzheimer's disease, depression and obesity, using antigens such as the amyloid protein, neurokinin 1 receptor or neuropeptide Y. Other preferred antigens include a GluR receptor (*e.g.*, GluR4, GluR6), an NPY neuropeptide, galanin, an NK-1 receptor, a dopamine transporter and glutamic acid decarboxylase, adenosine kinase, and neurokinin-1 (NK-1). Examples of molecules that can be used as antigens include, but are not limited to, receptors, transporters, ion channels, neurotransmitters, and the like.

(a) Receptors

The location of neurotransmitter receptors at synapses makes them a likely target for alterations during aging and in diseases that alter behavior and cognition. Examples of suitable receptors include, but are not limited to, N-methyl-D-aspartate (NMDA) receptor, neuronal glutamate receptors (GluR's), γ-aminobutyric acid receptors (GABAR's), nicotinic acetylcholine receptors, serotonin receptors, dopamine receptors, and the like. A preferred receptor is the NMDA receptor.

The NMDA is a class of glutamate receptor which is important in the pathology of many neurological disorders. Activation of NMDA receptors increases sodium and calcium conductance, which depolarizes the neuron, thereby increasing the likelihood that the neuron will fire an action potential. NMDA receptors are widely distributed throughout the brain, with a particularly high density in the cerebral cortex and hippocampal formation.

In the central nervous system, NMDA receptors are mediators of glutamatergic excitatory neurotransmission and are of major interest as they are involved in brain development including neuronal migration (Komuro *et al.* (1993) *Science* 260:95-97) patterning of afferent termination (Bear *et al.* (1990) *J. Neurosci.* 10:909-925) and several forms of long-term synaptic plasticity (Bliss and Collingridge (1993) *Nature* 361:31-39). Properties of the receptor include calcium permeability, voltage-dependent

Mg^{2+} block, and slow channel kinetics (McBain *et al.* (1994) *Physiol. Rev.* 74:723-760). Molecular cloning has revealed three receptor subunit families, NRI, NR3A and four types of NR2 subunits which in native NMDA receptor channels form hetero-oligomeric complexes (Hollmann and Heinemann (1994) *Ann. Rev. Neurosci.* 17:31-108; Das *et al.* (1998) *Nature* 393:377-381). NRI subunits are essential for the formation of functional NMDA receptors, whereas addition of other subunits modify receptor properties (Das *et al.* (1998) *Supra*; Sheng *et al.* (1994) *Nature* 368:144-147). In addition to the role of NMDA receptors in brain plasticity and development, they have also been implicated as a mediator of neuronal injury associated with many neurological disorders including stroke, epilepsy, brain trauma, AIDS dementia as well as neurodegenerative disorders (Beal (1992) *Current Opinion in Neurobiology* 2:657-662).

In another embodiment, the receptor is a glutamate receptor (GluR). Neuronal glutamate receptors (GluR's) comprise the predominant excitatory neurotransmitter system in the mammalian central nervous system (Choi (1992) *J. Neurobiol.* 23: 1261). Excessive glutamate receptor stimulation has been linked to subsequent neuronal death. This excitotoxicity is thought to play a role in nervous system destruction after stroke, trauma, epilepsy, Alzheimer's disease, and Huntington's disease. There are numerous subunits that compose the glutamate receptor family. Several subunits of the glutamate receptor have been molecularly cloned (*See e.g.*, Hollmann *et al.* (1993) *Ann. Rev. Neurosci.* 17: 31–108). These subunits are broadly grouped on the basis of sequence identity. These divisions include cDNAs that encode receptors with NMDA pharmacology and at least nine cDNAs that encode non-NMDA receptor types. This latter group can be subdivided into three groups based upon similarity of primary sequence and/or function. GluR1, GluR2, GluR3, and GluR4 type receptors that are responsive to kainic acid and α-amino-3-hydroxy-5-methyl-4- isoxazole propionate (AMPA) and bind AMPA with high affinity. GluR5, GluR6, and GluR7 type receptors that are responsive to kainic acid only, or bind kainic acid with high affinity.

(b) Neurotransmitters

Neurotransmitters are chemicals in the brain that are used to send messages from one brain cell to another. Neurotransmitters bind to special receptor proteins in the membranes of nerve cells, like a lock in a key, triggering a chemical reaction within the cell. Examples of neurotransmitters include, but are not limited to, dopamine, acetylecholine, and norepharine.

In one embodiment, the neurotransmitter is dopamine. Dopamine is an example of a central nervous system (CNS) neurotransmitter, and is a catecholamine belonging to a class of biogenic amine neurotransmitters, along with norepinephrine, serotonin, and histamine. The catecholamines (particularly dopamine and serotonin) are involved in the control of movement, mood, attention, and endocrine, cardiovascular, and stress responses. Imbalances in neurotransmitter production have been implicated in a variety of mental and physical disorders, such as Parkinson's disease, schizophrenia and psychosis.

Two major families of dopamine receptors have been identified and named the D1 and D2 families. In the D2 family, three distinct receptor subtypes have been identified as D2, D3, and D4. The distribution and concentration of the subtypes of receptors varies in different regions of the brain. D2 subtype receptors are located in both the limbic region of the brain, which is associated with cognition and emotional function, and in the stratium, which is associated with motor effects. D4 receptors are found in higher concentrations in the frontal cortex and limbic regions, which are associated with cognitive and emotional function.

In another embodiment, the neurotransmitter is acetylcholine (ACh) which activates two pharmacologically different receptor types: the nicotinic acetylcholine receptors (nAChR) from the ligand-gated ion channel superfamily, and the muscarinic acetylcholine receptors (mAChR) from the G-protein coupled receptor superfamily (Taylor *et al.* New York: Pergamon Press, (1990) 166-186; and 122-149). A number of pathologies and/or disease conditions are associated with nAChRs, for example, myasthenia gravis, schizophrenia, Alzheimer's disease, Tourette's disease and nicotine addiction.

In another embodiment, the neurotransmitter is serotonin. Serotonin is a hormone-neurotransmitter and has a role in physiologic processes such as sleep and in pathophysiologic conditions including depression, chronic pain, and migraine, and drug addiction. Serotonin is also a biosynthetic precursor of melatonin. Melatonin is important in temperature regulation, mood, and the sleep-wake cycles. Receptor binding sites for melatonin have been reported in discrete regions of the mammalian brain. Two mammalian melatonin receptors have been identified by expression cloning and shown to have expression patterns consistent with their predicted locations from hormone binding studies.

(c) Transporters

Certain amino acids, such as glutamate and glycine, as well as amino acid derivatives such as γ-aminobutyric acid (GABA), epinephrine and norepinephrine, and histamine, are also used as signaling molecules in higher organisms. For these reasons, specialized transmembrane transporter proteins have evolved in all organisms to recover or scavenge extracellular amino acids (for review *see* Christensen, (1990), *Physiol. Rev*. 70: 43-77).

These transporter proteins are important for the uptake of extracellular amino acids in the brain and peripheral motor and sensory tissues (*see* Nicholls & Attwell, (1990), *TiPS* 11: 462-468). Amino acids that function as neurotransmitters must be scavenged from the synaptic cleft between neurons to enable continuous repetitive synaptic transmission. High extracellular concentrations of certain amino acids (including glutamate and cysteine) can cause neuronal cell death, and are associated with a number of pathological conditions, including ischemia, anoxia and hypoglycemia, as well as chronic illnesses such as Huntington's disease, Parkinson's disease, Alzheimer's disease, epilepsy and amyotrophic lateral sclerosis (ALS: *see* Pines *et al.* (1992) *Nature* 360: 464-467).

Glutamate is an excitatory neurotransmitter (*i.e.*, excitatory neurons use glutamate as a neurotransmitter). When present in excess ($>$about 300 mM; Bouvier *et al.* (1992), *Nature* 360: 471-474; Choi *et al.*, (1987), *J. Neurosci.* 7: 357-358), extracellular glutamate causes neuronal cell death. Glutamate transporters play a pivotal role in maintaining non-toxic extracellular concentrations of glutamate in the brain. Glutamate transporters, for example, excitatory amino acid transporters (EAAT), can also be used in the present invention as antigens. Examples of suitable EAAT neurotransmitters, include but are not limited to EAAT1, EAAT2, EAAT3, EAAT4 and EAAT5 (*See e.g.*, U.S. patent No. 5, 919,628 issued to Amara *et al.*)

(d) Transcription factors

Polypeptides which can function as transcription factors to activate transcription in prokaryotic cells are well known in the art. Transcription factors comprise at least one DNA binding domain and at least one transcriptional activation domain (*e.g.*, parts of promoters or enhancer sequences). DNA binding domains that bind to specific regulatory sequences are also well known in the art (*see, e.g.*, Keegan *et al.* (1988), *Science*, 231, 699-704; Hope *et al.*(1986) *Cell*, 46, 885-894 and Ma *et al.* (1987) *Cell* 51, 113-119). Transcriptional activation domains found within various proteins have been grouped into categories based upon similar structural features. Types of transcriptional activation domains include acidic transcription activation domains, proline-rich transcription activation domains, serine/threonine-rich transcription activation domains and glutamine-rich transcription activation domains. Antigens of transcription factors may also be used in the invention.

In a preferred embodiment, the transcription factors are those implicated in neuronal activation. Transcription factors implicated in neurological disorders leading to cell death in neurons include c-fos, c-jun, and c-jun N-terminal kinase (JNK) in the in dopamine neurons of the substantia nigra (SN). The increased expression of c-jun is described to be functionally significant in the neuronal cell death, because it is associated with increased c-jun N-terminal kinase (JNK) and phosphorylated c-jun expression (Oo *et al.* (1999) *J. Neurochem.* 72:557-64 and Chihab *et al.* (1998) *Brain Res Mol Brain Res* 63:105-120). Other examples of transcription factors important in neurological disorders include protein kinase C (PKC) activity. For example, increased presynaptic protein kinase C activity is associated with by increased glutamate release (Di Luca *et al.* (1997) *Eur. J. Neurosci.* 9:472-479).

(e) Growth Factors

In another embodiment, the antigen can be a growth factor which can stimulate or retard cell growth. Studies have shown in animal models of neurodegenerative diseases, that delivering a neurotrophic factors, such as nerve growth factor (NGF), which sustains the growth and development of neurons, prevents damage-induced death, and attracts the growth of developing or regenerating axons, to the area of neurodegeneration. Suitable examples of growth factors include, but are not limited to, glial cell line-derived neurotrophic factor (GDNF), ciliary derived neuronotrophic factor (CNTF), brain derived neuronotrophic factor (BDNF), neuronotrophin-3 (NT3), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF),

(f) Ion channel Proteins

Ion channel proteins are mediators of information transfer in the brain, endocrine system, enteric nervous system and neuromuscular junction, modulate ion fluxes that produce voltage changes across cell membranes and simultaneously act as sensors of physiological signals, for example, changes in ligand concentrations and in transmembrane voltage. Ligand-gated ion channels provide a rapid dialogue between cells of the central nervous system, converting a chemical neurotransmitter signal released from one cell into an electrical signal that propagates along the cell membrane of a target cell. Ligand-gated ion channels are multimeric protein complexes with component subunits encoded by related genes.

Numerous families of ligand-gated receptors have been identified and characterized on the basis of sequence identity. Those which form cationic channels include, for example, excitatory nicotinic acetylcholine receptors (nAChRs), excitatory glutamate-activated receptors, the 5-HT_3 serotonin receptor, the ATP receptor and the sarcoplasmic ryanodine receptor. Those which form anionic channels include, for example, the inhibitory GABA and glycine-activated receptors.

(g) Signal transduction molecules

Reversible phosphorylation of proteins is a prevalent biological mechanism for modulation of enzymatic activity in living organisms. (Tonks *et al.* (1988), *J. Biol. Chem.* 263:6722-6730). Such reversible phosphorylation requires both a protein kinase (PK), to phosphorylate a protein at a particular amino acid residue, and a protein phosphatase (PP), to remove the phosphate moieties. *See e.g.*, Hunter,(1995) *Cell,* 80:225-236. One major class of protein kinases are the serine/threonine kinases and a major type of protein phosphatases are protein serine/threonine phosphatases. These protein kinases and phosphatases have been shown to play critical roles in the regulation of metabolism. (*See e.g.*, Cohen, (1992) *Trends Biochem. Sci.*, 17:408-413; Shenolikar, (1994) *Ann. Rev. Cell Biol.*, 10:55-86; Bollen *et al.* (1992), *Crit. Rev. Biochem. Mol. Biol.* 27:227-281). These enzymes phosphorylate and dephoshphorylate serine and threonine residues of substrate proteins. Preferably, the serine/threonine kinases or serine/ threonine phosphatases are those that are involved in neuronal activation.

Another group of protein kinases and phosphatases includes the tyrosine kinases an tyrosine phosphatases. The protein tyrosine kinases and the protein tyrosine phosphatases comprise enzymes that have been implicated in the control of normal and neoplastic cell growth and proliferation. *See* Fisher *et al.* (1991), *Science,* 253:401-406. Protein tyrosine kinase (PTK) genes share a high degree of inter-species conservation. (*See e.g.*, Hunter and Cooper, (1985) *Ann. Rev. Biochem.* 54:897-930. PTK enzymes exhibit high specificity for tyrosine, and ordinarily do not phosphorylate serine, threonine, or hydroxyproline. Their roles in cellular processes include, cell-cell contact and cell adhesion, and growth factor and antigen signaling events. Preferably, the tyrosine kinases or tyrosine phosphatases are those that are involved in neuronal activation.

II. Antibodies

Antibodies to the antigens for direct transfer of immunity can also be administered. Antibodies can be generated using standard techniques known in the art and include recombinant antibodies, chimeric antibodies, humanized antibodies, and the like. The antibody may be of animal, *e.g.*, a mouse or rat. Preferably, the antibody is a human antibody. The antibody may be a chimeric antibody (*See e.g.*, Morrison *et al.*, (1984) *Proc Nat. Acad. Sci. U.S.A.* 81: 6851-6855) or a humanized antibody (*See e.g.*, Jones *et al.* (1986) *Nature* 321: 522-525. Methods of producing antibodies suitable for use in the present invention are well known to those skilled in the art and can be found described in such publications as Harlow & Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1988), and Asai, Methods in Cell Biology Vol. 37. Antibodies in Cell Biology, Academic Press, Inc. N.Y. (1993).

When antibodies are generated by immunizing animals with an antigen to yield antibody which is not of human origin, the antibodies could elicit an adverse effect if administered to humans. Methods for "humanizing" antibodies, or generating less immunogenic fragments of non-human antibodies, are well known. A humanized antibody is one in which only the antigen-recognized sites, or complementarity--determining hypervariable regions (CDRs) are of non-human origin, whereas all framework regions (FR) of variable domains are products of human genes. These "humanized" antibodies present a lesser xenografic rejection stimulus when introduced to a human recipient.

To accomplish humanization of a selected mouse monoclonal antibody, the CDR grafting method described by Daugherty *et al.*(1991) *Nucl. Acids Res.* 19:2471-2476, may be used. Briefly, the variable region DNA of a selected animal recombinant anti-idiotypic ScFv is sequenced by the method of Clackson, *et al.* (1991), *Nature*, 352:624-688. Using this sequence, animal CDRs are distinguished from animal framework regions (FR) based on locations of the CDRs in known sequences of animal variable genes (*See e.g.*, Kabat, *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). Once the animal CDRs and FR are identified, the CDRs are grafted onto human heavy chain variable region framework by the use of synthetic oligonucleotides and polymerase chain reaction (PCR) recombination. Codons for the animal heavy chain CDRs, as well as the available human heavy chain variable region framework, are built in four (each 100 bases long) oligonucleotides. Using PCR, a grafted DNA sequence of 400 bases is formed that encodes for the recombinant animal CDR/human heavy chain FR protection.

For recombinant production of antibodies, the sequences of human heavy chain constant region genes are known in the art (*See e.g.*, Kabat, *et al. Supra*) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region and any allotypic variant therein as described in Kabat *et al.*, *supra* The sequences of human light chain constant region genes are known in the art and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, *e.g.*, encoding the amino acid sequence (Gly_4-$Ser)_3$, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (*See e.g.*, Bird *et al.* (1988) *Science* 242:423-426; Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; McCafferty *et al.* (1990) *Nature* 348:552-554). To express the antibodies, or antibody portions, DNAs encoding partial or full-length light and heavy chains are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (*e.g.*, ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell.

The recombinant expression vectors used for antibody production carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; (1990) *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA. Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP)) and polyoma.

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains can be transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in Kaufman *et al.* (1982) *Mol. Biol.* 159:601-621), NSO myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments or scFv molecules. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than NMDA by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

III. Pharmaceutical Compositions and Pharmaceutical Administration

The antigen, antibodies or antigen-binding portions of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises an antigen, antibody or antibody portion of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody or antibody portion.

The antigens, antibodies and antibody-portions of the invention can be incorporated into a pharmaceutical composition suitable for parenteral administration. Other suitable buffers include but are not limited to, sodium succinate, sodium citrate, sodium phosphate or potassium phosphate. Sodium chloride can be used to modify the toxicity of the solution at a concentration of 0-300 mM (optimally 150 mM for a liquid dosage form). Cryoprotectants can be included for a lyophilized dosage form, principally 0-10% sucrose (optimally 0.5-1.0%). Other suitable cryoprotectants include trenhalose and lactose. Bulking agents can be included for a lyophilized dosage form, principally 1-10% mannitol (optimally 2-4%). Stabilizers can be used in both liquid and lyophilized dosage forms, principally 1-50 mM L-Methionine (optimally 5-10 mM). Other suitable bulking agents include glycine, arginine, can be included as 0-0.05%

polysorbate-80 (optimally 0.005-0.01%). Additional surfactants include but are not limited to polysorbate 20 and BRIJ surfactants.

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (*e.g.*, injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans. The preferred mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antigen, antibody or antibody-portion is administered by intravenous infusion or injection. In another preferred embodiment, the antibody is administered by intramuscular or subcutaneous injection. In the most preferred embodiment, the antigen, antibody or antibody portion is administered perorally.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (*i.e.*, antigen, antibody or antibody portion) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization.

Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile, lyophilized powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and spray-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

The antigen, antibody or antibody portion of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. *See, e.g.*, *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

In certain embodiments, an antigen, antibody or antibody portion of the invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antigen, antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents that are useful for treating disorders in which NMDA receptor activity is detrimental. For example, an anti-NMDA antibody or antibody portion of the invention may be coformulated and/or coadministered with one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface molecules). Furthermore, one or more antigens or antibodies of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies.

Antigens, antibodies or antibody-portion can be used alone or in combination to treat diseases. For example, the antigen, antibodies, or antibody portion can be used alone or in combination with an additional agent, *e.g.*, an agent which imparts a beneficial attribute to the therapeutic composition *e.g.*, an agent which effects the viscosity of the composition. The combination can also include more than one additional agent, *e.g.*, two or three additional agents if the combination is such that the formed composition can perform its intended function.

The invention is also intended to include one or more combinations of antigens, such that one or more antibodies are produced against the antigens in the systemic circulation of the subject. For example, the first antigen can a portion of the NMDAR1 receptor, and the second antigen can be a portion of the Glu R receptor. Accordingly, antibodies to both NMDAR1 and Glu R receptor can be produced. The skilled artisan will appreciate that any combination of one or more antigen can be used to produce the genetic vaccine of the invention.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of an antigen, antibody or antibody portion of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antigen, antibody or antibody portion may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody or antibody portion to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody or antibody portion are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antigen, antibody or antibody portion of the invention is 0.1-20 mg/kg, more preferably 1-10 mg/kg. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

IV Delivery Systems

The invention features a method of using adeno-associated viral vectors (AAV) comprising the NMDA antigen in a genetic vaccine. AAV vectors can be constructed using known techniques to provide at least the operatively linked components of control elements including a transcriptional initiation region, a exogenous nucleic acid molecule encoding an antigen, and a transcriptional termination region. The control elements are selected to be functional in the targeted cell. The resulting construct which contains the operatively linked components can be flanked at the 5' and 3' region with functional AAV ITR sequences.

The preferred AAV is AAV-2 as described by Kotin *et al.* (1994) *Human Gene Therapy* 5:793-801; Berns "Parvoviridae and their Replication" in Fundamental Virology, 2nd Edition, (B. N. Fields and D. M. Knipe, eds.), although other AAV serotypes can be used in the invention. Examples of other AAV serotypes include, but not limited to, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAVX7, and the like.

Control sequences can often be provided from commonly used promoters derived from viruses such as, polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. Use of viral regulatory elements to direct expression of the protein can allow for high level constitutive expression of the protein in a variety of host cells. Ubiquitously expressing promoters can also be used include, for example, the early cytomegalovirus promoter Boshart *et al.* (1985) *Cell* 41:521-530, herpesvirus thymidine kinase (HSV-TK) promoter (McKnight *et al.* (1984) *Cell* 37: 253-262), β-actin promoters (*e.g.*, the human β-actin promoter as described by Ng *et al.* (1985) *Mol. Cell Biol.* 5: 2720-2732) and colony stimulating factor-1 (CSF-1) promoter (Ladner *et al.* (1987) *EMBO J.* 6: 2693-2698). Alternatively, tissue-specific regulatory elements can be used, such as tissue specific promoters.

In another embodiment, the vector of the invention can be a virus other than the adeno-associated virus, which allows for expression of a nucleic acid molecule introduced into the viral nucleic acid. For example, replication defective retroviruses, adenoviruses and lentivirus can be used. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel *et al.* (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. The genome of adenovirus can be manipulated such that it encodes and expresses the protein of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. *See e.g.*, Berkner *et al.* (1988) *BioTechniques* 6:616; Rosenfeld *et al.* (1991) *Science* 252:431-434; and Rosenfeld *et* *al.* (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (*e.g.*, Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art.

Delivery systems include methods of *in vitro*, *in vivo* and *ex vivo* delivery of the antigen, antibody or antigen-binding portion. Generally, the antigen is delivered to the systemic circulatory system using methods known in the art. Preferred methods include peroral administration of the antigen. Other methods include intramuscular injection of the antigen, as discussed in section III. Antibodies can also be administered to the systemic system or directly to a targeted site in the region of the brain.

For *in vivo* delivery of antigen or antibodies or an antigen-binding portion, the antigen or antibody can be administered to a subject in a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier", as used herein, refers to any physiologically acceptable carrier for *in vivo* administration of the vectors of the present invention. Such carriers do not induce an immune response harmful to the individual receiving the composition, and are discussed in section III.

In one embodiment, antibody or antibody portion can be distributed throughout a wide region of the CNS, by injecting the antibody or antibody portion into the cerebrospinal fluid, *e.g.*, by lumbar puncture (*See e.g.*, Kapadia *et al.* (1996) *Neurosurg* 10: 585-587).

Alternatively, precise delivery of the antibody into specific sites of the brain, can be conducted using stereotactic microinjection techniques. For example, the subject being treated can be placed within a stereotactic frame base (MRI-compatible) and then imaged using high resolution MRI to determine the three-dimensional positioning of the particular region to be treated. The MRI images can then be transferred to a computer having the appropriate stereotactic software, and a number of images are used to determine a target site and trajectory for antibody microinjection. The software translates the trajectory into three-dimensional coordinates that are precisely registered for the stereotactic frame. In the case of intracranial delivery, the skull will be exposed, burr holes will be drilled above the entry site, and the stereotactic apparatus used to position the needle and ensure implantation at a predetermined depth. The antibody, or antibody portion can be delivered to regions, such as the cells of the spinal cord, brainstem, (medulla, pons, and midbrain), cerebellum, diencephalon (thalamus, hypothalamus), telencephalon (corpus stratium, cerebral cortex, or within the cortex, the occipital, temporal, parietal or frontal lobes), or combinations, thereof.

Alternatively, the antigen, antibody, or antibody portion, can be delivered using a non-viral delivery system. This includes delivery of the antigen or antibody or antibody portion to the desired tissues in colloidal dispersion systems that include, for example, macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes.

Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genetic material at high efficiency while not compromising the biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al.* (1988) *Biotechniques*, 6:682). Examples of suitable lipids liposomes production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Additional examples of lipids include, but are not limited to, polylysine, protamine, sulfate and 3b -[N- (N',N' dimethylaminoethane) carbamoyl] cholesterol.

Alternatively, the antigen can be administered as a peptide vaccine. A synthetic peptide comprising an antigen binding region can be prepared using standard peptide synthesis method known in the art. It is often necessary to couple the peptide with a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and human serum albumin. Other carriers may include a variety of lymphokines and adjuvants such as INF, IL2, IL4, IL8 and others. Means for conjugating a peptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimyde and bis-biazotized benzidine. It is also understood that the peptide may be conjugated to a protein by genetic engineering techniques that are well known in the art. The preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art. (*See e.g.*, U.S. Pat. Nos. 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770).

In one embodiment, particle-mediated delivery using a gene-gun can be used as a method to vaccinate a subject. Suitable particles for gene gun-based delivery of include gold particles which are coated with the DNA encoding the antigen. In one embodiment, the DNA encoding the antigen can be delivered as naked DNA without an expression vector. In another embodiment, the DNA encoding the antigen can be inserted into an expression plasmid. The antigen coated particles can be delivered into the epidermal layer of the skin to elicit an inflammatory response. Gene gun based delivery is described, for example by, Braun *et al.* (1999) *Virology* 265:46-56; Drew *et al.* (1999) *Vaccine* 18:692-702; Degano *et al.* (1999) *Vaccine* 18:623-632; and Robinson (1999) *Int J Mol Med* 4:549-555; Lai *et al.* (1998) *Crit Rev Immunol* 18:449-84;*See e.g.*, Accede *et al.* (1991) *Nature* 332: 815-818; and Wolff *et al.* (1990) *Science* 247:1465-1468 Murashatsu *et al.*, (1998) *Int. J. Mol. Med.* 1: 55-62; Agracetus *et al.* (1996) *J. Biotechnol.* 26: 37-42; Johnson *et al.* (1993) *Genet. Eng.*15: 225-236).

Expression of administered genes results in the induction of humoral and cellular immune responses against the encoded antigen. The nature of the immune response depends on the route, method, and timing of DNA delivery and can also be influenced by co-delivery of plasmids encoding immunomodulating cytokines like IFN-alpha, IL-2, or IL-12 and costimulatory molecules like B7-1 (*See e.g.*, Tuting (1998) *J Invest Dermatol* 111:183-188 and Barry *et al.* (1997) *Vaccine* 15:788-791). The method of DNA inoculation (gene gun versus intramuscular injection) and the form of the DNA-expressed antigen (cell-associated versus secreted) determine whether T-cell help will be primarily type 1 or type 2. Mechanistically, gene gun-delivered DNA initiates responses by transfected or antigen-bearing epidermal Langerhans cells that move in lymph from bombarded skin to the draining lymph nodes. Following intramuscular. injections, the functional DNA appears to move as free DNA through blood to the spleen where professional antigen presenting cells initiate responses (Robinson *et al.* (1997) *Semin Immunol* 9:271-283).

Also within the scope of the invention is the delivery of the antigen in one or more combinations of the above delivery methods. For example, intradermal delivery of an antigen, followed by intramuscular injection of the antigen.

V Functional Genomics

In another embodiment, the antigen, antibody or antibody portion of the invention can be used to modify the expression and/or secretion of one or more molecules (*e.g.*, a neurotrophic factor, a neurotransmitter, or a neuroprotective agent), *e.g.*, to enhance their neuroprotective capacity. For example, in order to provide neuroprotection in epilepsy or stroke, the NMDAR1 antigen can be administered to a subject to produce antibodies in the systemic circulatory system of the subject. The circulating anti-NMDAR1 antibodies migrate across the blood-brain barrier into the cerebrospinal fluid and bind to the NMDAR1 receptors in the cortex upon injury, disease or excessive neuronal activity. Migration of the antibodies into the cerebrospinal fluid as a result of kainate induced injury, is shown in Fig. 5A and in Fig. 10I. Example 6 demonstrates direct modulation of the NMDR receptor upon antibody binding. An indirect effect of antibody binding is also demonstrated based on monitoring Krox-24 expression. These binding of the anti-NMDAR1 to the NMDA receptors upregulates their expression, as demonstrated by the increase in their mRNA expression (*See* Example 6).

Accordingly, the invention also provides for methods of modifying the function of a target gene. The term "target protein" as used herein refers to a molecule to which an antibody binds and changes the function of. For example, the NMDA receptor to which the anti-NMDAR1 antibody binds, is a target protein. Modification of the target protein can, for example, increase, decrease, elevate or depress the secretion of certain molecules, for example, a neurotrophic factor, an altered expression of proteins (*i.e.* increase or decrease in protein expression) and modifications in the morphological and functional processes.

The invention also provides a method of introducing a new function to a target cell (*e.g.*, a damaged neural cell) in a phenotypically useful way. A new function can be expressed in such defective target cells (*e.g.*, damaged neural cells) by modifying the function of a target protein in/or on a cell. For example, by decreasing expression of the Knox-24 protein or by increasing the number of NMDA receptors (*See* example 3). Such modifications can result in a gain-of-function response, for example, improved learning and memory (*See* Example 7).

In one embodiment, modifications to cells using the methods of the invention includes the changes to the expression and/or secretion of a gene product. For example, to augment neurotransmitter function within the brain, such as, modifications to increase or decrease expression of choline acetyltransferase. Another example, includes modifications to produce tyrosine hydroxylase (an enzyme that coverts tyrosine to L-DOPA). The antigen or antibody of the invention can by used to increase the production of this enzyme and to continue to convert tyrosine to L-DOPA in the striatum. In a preferred embodiment, the modification can alter NMDA receptor numbers directly, or decrease Knox-24 protein expression (*See* Example 6). These modifications to the target gene can be used for functional genomic studies. For example, to modulate the effect of proteins and substances such as receptors (*e.g.* NMDA, GluR); neurotransmitters (*e.g.*, dopamine, acetylcholine, serotonin, histamine and melatonin); transporters (*e.g.*, EAAT); transcription factors, growth factors (*e.g.*, epidermal growth factor, brain derived neurotrophic growth factor); ion channel proteins; and signal transduction molecules, as described in section I.

VI. Diseases

The brain, retina and testis are generally perceived as immune privileged sites as defined traditionally by the prolonged survival of allogeneic or xenogeneic tissue transplanted into these organs. Although it is now known that this immune privilege is relative and not absolute, many foreign antigens within the CNS escape immune surveillance and, moreover, self-antigens in the brain may not induce tolerance. Studies have shown that in paraneoplastic disorders (PND), circulating autoantibodies interact with neuronal antigens (Posner and Furneaux 1990. *Immunologic Mechanisms in Neurologic and Psychiatric Disease*, ed. Waksman, B. H., 187-219). It is believed that brain antigens previously sequestered from the immune system via the blood-brain barrier become presented to a naïve immune system when ectopically expressed in cancer cells and elicit a humoral immune response (Darnell, (1996) *Proc Natl Acad Sci*

USA, 93:4529-4536).

Circulating plasma proteins and other large non-lipophilic molecules have very poor access to brain because of the impermeability of the cerebral endothelial tight junctions and astrocytic foot processes which constitute the blood-brain barrier (blood-brain barrier). Antigens within brain parenchyma generally undergo less immunosurveillance and antibodies circulating in the blood cross this barrier poorly under normal, basal conditions (Pollack and Lund (1990) *Exp. Neurol.* 108:114-121). There are examples which suggest that this immune privilege is relative, not absolute, and that the nervous system is not protected from antibody-mediated attack and protection. For example, the myasthenic syndrome of Lambert Eaton is associated with circulating antibodies which bind to nerve terminals, the principal target being the P/Q-type voltage-gated calcium channel (Kim and Neher (1988) *Science* 239:405-408). Circulating antibodies have also been described in Stiffinan syndrome (Solimena *et al.* (1988) *New England Journal of Medicine* 318:1012-1020) and Rasmussen's Disease (Rogers *et al.* (1994) *Science* 265:648-651). It is established that antibodies pass the blood-brain barrier poorly (Pollack and Lund (1990) *Exp. Neurol* 108:114-121), however, the blood-brain barrier is compromised after insults to the brain, including trauma, seizures, stroke or infection, allowing penetration of plasma molecules, including antibodies, into brain parenchyma.

While not required to provide a mechanism of action, the genetic vaccine of the invention may provide neuroprotective effects when the integrity of the blood-brain barrier is compromised (*e.g.*, due to insult or injury to the brain, disease or excessive neuronal activity). The compromise in the blood-brain barrier enables a breach of the immune privilege of the brain and passage of antibodies to the targeted neurons resulting in the characteristic disease phenotype. Accordingly, the invention features a method of treating neurological disorders by vaccinating against selected brain antigens to induce a state of autoimmunity. An immune response to a brain self-antigen can be induced which, instead of having disease-inducing activity, has a therapeutic efficacy. Examples 3 and 4 demonstrate the neuroprotective effect of a vaccine comprising an NMDA receptor antigen that elicited the production of antibodies against the NMDA receptor. The genetic vaccine of the invention can be used to induce a high titer of circulating NMDA receptor autoantibodies which have minimal CNS penetration under resting, basal conditions. However, following an insult, injury, disease or excessive neuronal activity, to the brain and compromise of the blood-brain barrier, these antibodies pass into the brain more efficiently, bind to and interfere with the function of the NMDA receptor, thereby attenuating NMDA receptor-mediated injury.

In one embodiment, the invention provides a method for inhibiting NMDA activity in a subject suffering from a disorder in which NMDA receptor activity is detrimental. The NMDA receptor has been implicated in the pathophysiology of a wide variety of disorders. The invention provides methods for modulating NMDA activity in a subject suffering from such a disorder, which method comprises administering to the subject an antigen, antibody or antibody portion, such that NMDA receptor activity in the subject is modulated. Preferably, the subject is a human subject.

A disorder in which NMDA receptor activity is detrimental is a disorder in which modulation of NMDA receptor activity is expected to alleviate the symptoms and/or progression of the disorder. Antigens, antibodies, or antibody portions can be introduced into a subject in an amount suitable to ameliorate, reduce, alleviate or aid in, or at least partially correct a neurological disorder. Alleviation of the disorders may be evidenced, for example, by the neuroprotective effect of the antibody or antibody binding portion on regions of the brain (*See* Example 3 for neuroprotection against epilepsy and Example 4, for neuroprotection against stroke, and by the behavioral test described in Example 1).

There are numerous examples of disorders in which NMDA receptor activity can be detrimental. Neurological disorders to be treated by the invention include, but are not limited to, epilepsy, stroke, Parkinson's disease, Alzheimer's and other disorders in which the disease process is in part mediated by a brain protein or where a molecule binding to a brain protein would alter the disease phenotype, for example proteins involved in the signal transduction of neurotransmitters including receptors and ion channels, or the synthesis of neurotransmitters or the uptake and transport of brain chemicals. Representative examples of neurotransmitter receptors include, but are not limited to, the NMDA, AMPA and kainate receptors, dopamine, serotonin and noradrenergic receptors and transporters and neuropeptide receptors including the neurokinin-1 (NK1) receptor. Representative examples of transmitters include, but are not limited to, glutamate, GABA, dopamine, serotonin, acetylcholine, norepinephrine, adenosine, neuropeptide Y (NPY) and substance P. Representative examples of proteins that are important to neurological disorder include, but are not limited to, amyloid protein (AP) and amyloid precursor protein (APP) as well as the CAG repeat protein, huntington.

In one embodiment, the antigens, or antibodies or antibody portion thereof, can be used in therapy to treat the diseases or disorders described herein. In another embodiment, the antigen, antibodies or antibody portions thereof, can be used for the manufacture of a medicine for treating the diseases or disorders described herein. The use of the antigens, antibodies and antibody portions of the invention in the treatment of a few non-limiting specific disorders is discussed further below:

Stroke

In a preferred embodiment, the neurological disorder is stroke. A vaccine compositions comprising antigens of the NMDA receptor, in particular, antigens to NMDAR1 subunit, can be used to generate antibodies in the systemic circulation of a subject, as described in detail Example 3. The circulating antibodies penetrate the blood-brain barrier to provide a neuroprotective effect against stroke.

Epilepsy

In another preferred embodiment, the neurological disorder is epilepsy. In the case of epilepsy, there are both rat and monkey models in which effective therapies are predictive of therapeutic efficacy in humans. For example, rats which exhibit audiogenic seizures are commercially available. Example 4 demonstrates in detail the neuroprotective effect of the AAVNMDAR1 vaccine against seizures in the kainate epilepsy model. Antigen, antibodies or antibody portions of the invention can be introduced into the systemic circulation of these animals and seizures initiated. The neuroprotective capacity of the vaccine was determined by monitoring the onset, or decrease in seizure occurrence by EEG. Fig. 4A shows the reduction in epileptic seizures in animals vaccinated with the AAVNMDAR1 vaccine of the invention.

Huntington's disease

The antigen or antibody or antibody portion can be used to treat neurological disorders resulting from neurodegeneration, such as that which occurs in human subjects with Huntington's disease. Models of neurodegenerative diseases in several different animals have been developed. For example, rat (Isacson *et al.* (1985) *Neuroscience* 16:799-817), monkey (Kanazawa, *et al.* (1986) Neurosci. Lett. 71:241-246), and baboon (Hantraye. *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:4187-4191; Hantraye,. *et al.* (1990) *Exp. Neurol.* 108:91-014; Isacson, *et al.*(1989) *Exp. Brain Res.* 75(1):213-220) models of Huntington's disease have been described in which effective therapies are predictive of therapeutic efficacy in humans. Neurodegeneration in Huntington's disease typically involves degeneration in one or both nuclei forming the stratium or corpus stratium, the caudate nucleus and putamen. Administration of the antigen to the systemic circulatory system may result in antibodies to specific receptors or proteins in these regions. These antibodies may enter the central nervous system upon injury due to neurodegeneration in these regions and bind to a target gene to offer neuroprotection. Alternatively, antibodies or antibody portions generated in a mammal can be introduced into the systemic circulatory system, or to specific affected brain regions.

To assess therapeutic strategies, the antigen or antibody of the invention can be introduced into the animal model and a state resembling Huntington's diseases can be generated. Morphological and immunohistochemical studies can then be performed by conventional techniques to determine whether the antibody provided neuroprotection by assessing, both morphologically and functionally of the tissue. Behavioral tests can also be performed using standard techniques (*See* Example 1).

Parkinson's disease

Parkinson's disease in humans primarily affects subcortical structures, especially the substantia nigra and loercus caeruleus. It is characterized by the loss of dopamine neurons in the substantia nigra, which have the basal ganglia as their major target organ. Several animal models of Parkinson's disease have been generated in which effective therapies are indicative of therapeutic efficacy in humans. These animal models include three rat models (the rats having lesions in substantia nigral dopaminergic cells caused by treatment with 6-hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or surgical transection of the nigral striatal pathway) (*See, e.g.* Björklund *et al.* (1982) *Nature* 298:652-654), a rhesus monkey model (the monkeys having lesions in substantia nigral dopaminergic cells caused by treatment with MPTP) (*See, e.g.*, Smith, *et al.* (1993) *Neuroscience* 52):7-16; Bakay *et al.* (1985) *Appl. Neurophysiol.* 48:358-361; Zamir. *et al.* (1984) *Brain Res.* 322:356-360), and a sheep model (the sheep having lesions in substantia nigral dopaminergic cells caused by treatment with MPTP) (Baskin, *et al.* (1994) *Life Sci.* 54:471-479). In another embodiment, the antigen, antibody or antibody portion of the invention can be used to treat a subject with Parkinson's disease. To assess therapeutic strategies, morphological and immunohistochemical studies can be performed by conventional techniques. Behavioral tests can also be performed (*See* Example 1).

Amyloid Lateral Sclerosis (ALS)

Several models of amyloid lateral sclerosis are available. Mutations in the superoxide dismutase gene 1 (SOD-1) are found in patients with familial amyotrophic lateral sclerosis (FALS). Overexpression of a mutated human SOD-1 gene in mice results in neurodegenerative disease as result of motor neuron loss in lumbar spinal cord, providing a suitable model for FALS (*See e.g.*, Mohajeri *et al.* (1998) *Exp Neurol* 150:329-336). Transgenic models of ALS are also described (*See e.g.*, Gurney (1997) *J Neurol Sci* 152:S67-73). Expression of mutant SOD1 genes in transgenic mice causes a progressive paralytic disease whose general features resemble ALS in humans. These models can be used to examine the effect of an antigen, antibody or antibody portion that can be used to modify the function of receptors or transporter proteins associated with ALS (*e.g.*, EAAT2 transporter protein). A gain-of-function in these models can monitored, for example, improvement in motor impairments of the animal's limbs.

Neuronal activation has also been associated with a number of neuroendocrine systems. Different regions of the brain activate different neuroendocrine systems (Hoffman *et al.* (1993) *Front Neurendocrinol.* 14: 173-213) and physiological response injury (Dubner *et al.* (1992) *Trends Neurosci.* 15: 96-103). Accordingly, the invention also provides methods and compositions of treating neuroendocrine disorders, for example, obesity and diabetes mellitus.

Obesity

The antigen, antibodies or antibody portions can also be used to treat or modify obesity in a subject. Mouse models for obesity are known in that art, for example, obese-diabetic mice (ob/ob), and and obese-diabetic (db/db) mice from the Jackson Laboratories (Bar Harbor, Me). (*See e.g.*, Collins *et al.* (1996) *J Biol Chem* 271:9437-9440; Darling (1996) *Curr Opin Genet Dev* 6:289-294; Andersson (1996) *Ann. Med.* 28:5-7; leptin (Van Heek *et al.* (1997) *J. Clin. Invest* 99:385-390). These animal models can be used to assess the effect of an antigen, antibody or antibody portion on weight gain by modifying the function of neurotransmitters.

The hypothalamus plays a significant role in obesity, particularly in regulating neuropeptide Y (NPY), a 36 amino acid peptide secreted by hypothalamic neurons and a potent substance that stimulates appetite. NPY belongs to a family of neuroendocrine peptides including pancreatic polypeptide and peptide YY. The amino acid sequence of NPY and the location of NPY-expressing neurons within the brain are described in Larhammar, (1996) *Regulatory Peptides*, 62:1-11. NPY is observed in the hypothalamus of obese animals (Sanacora *et al.*, (1990) *Endocrinol.*127:730-737; Sanacora *et al.* (1990) *J Neuroendocrinol.*, 4:353-357). Secretion of NPY from neurons within the hypothalamus stimulates feeding and chronically high levels of NPY expression result in hyperphagia and obesity. The ability to reduce high levels of NPY results in the diminution of the drive to eat. NPY gene regulation and physiology are reviewed in Berelowitz *et al.* (1992) *TEM* 3:127-133.

Growth hormone releasing factor (GRF), is another example of a peptide present in high concentration in the hypothalamus. GRF is the primary stimulatory factor controlling synthesis and secretion of pituitary growth hormone (GH), a critical regulatory hormone of metabolic homeostasis controlling breakdown of fat (lipolysis) and synthesis of protein. The antigen, antibody or antibody portion can be used regulate, the expression of neurotransmitters such as neuropeptide Y. Examples of other neurotransmitters involved in feeding and metabolism include, galanin, norepinephrine, dopamine, and β-endorphin release. Additional examples include, but are not limited to, cocaine-and amphetamine-regulated transcript (CART), orexin, thyrotropin-releasing hormone (TRH), leptin, corticotropin-releasing hormone (CRH) and pro-opiomelanocortin (POMC). The antigen, antibody or antibody portion can also be used to modify the receptors of these neurotransmitters.

Diabetes

A summary of insulin-dependent diabetes mellitus and its animal models is described by Wong *et al.* (1999) *Curr Opin Immunol* 11:643-647. These models can be used to investigate the effect of an antigen, antibody or antibody portion on diabetes in a animal. A few autoantigens have been associated with Type I diabetes mellitus, for example, insulin (Palmer *et al.* (1983) *Science* 222:1337-1339), glutamic acid decarboxylase (GAD) (Baekkeskov *et al.* (1990) *Nature* 347:151-156) and carboxypeptidase H (Castano. *et al.* (1991) *J. Clin. Endocr. Metab*, 73:1197-1201), and the glycolipids GT3 (Gillard, *et al.* (1989) *Journal Immunol. Methods* 142:3826-3832) and GM2-1 (Dotta, *et al.* (1992) *Endocrinology* 130:37-42) and PM-1 (U.S. 5,908,627). The methods of the invention to treat or prevent the development of Type I diabetes by modulating these proteins.

Reproduction

Substances involved in reproduction can also be modified by using antigen, antibodies, or antibody portions to modify the function of these substances. Suitable animal models for reproduction are Sprague-Dawley rats, which are readily available. For example, modifying the function of luteinizing hormone-releasing hormone (LHRH), a hormone regulated by the hypothalamus and involved in the stimulation of ovulation and uterine growth (Fueshko *et al.* (1994) *Dev Biol* 166:331-348;Hahn *et al.* (1984) *Endocr Res*, 10:123-138). Luteinizing hormone-releasing hormone also plays a role in male sterility by inhibiting the action of luteinizing hormone-releasing hormone with a synthetic decapeptide (Carelli (1982) *Proc Natl. Acad. Sci. U S A* 79:5392-5395).

Another substance with a role in reproduction is colony-stimulating factor-1 and the CSF-1 null mouse model can be used to study the biological functions of CSF-1. CSF-1 is a neurotrophic factor acting through the microglia and the absence of CSF-1 results in severe electro-physiological abnormalities in the cortex (Pollard (1997) *Mol Reprod Dev* 46:54-60). A role for CSF-1 in reproduction was originally suggested by the sex steroid hormone-regulated uterine epithelial synthesis of CSF-1 and the expression of its receptor in trophoblast and decidual cells. CSF-1 also show that it functions in male fertility.

The method of the invention can be used to treat disorders, such as mania, anxiety, depression and psychosis (*See* PCT Nos. WO 95/16679, WO 95/18124 and WO 95/23798). Neurokinin-1 (NK-1) receptor plays a significant role in these disorders. Neurokinin-1 antagonists are being developed for the treatment of a number of physiological disorders associated with an excess or imbalance of tachykinins, and in particular substance P. The method of the invention can also be used to produce antibodies against NK-1 to treat bipolar disorders.

Depression

The antigen, antibodies or antibody portions can also be used to treat or alleviate depression. A well known animal model for depression is the Porsolt's swim test model (Porsolt, (1979) *Biomedicine* 30:139-140). This model investigates the behavior of rats or mice when forced to swim in a restricted space and the attempts of the animals to escape and become immobile. The immobile state reflects the state of lowered mood in the animal. This model can be used to investigate the effect an antigen, antibody or antigen on neuropeptides, neurotransmitters and receptors involved in depression, for example, NK-1, the effect of brain histamine and histamine receptors (Lamberti *et al.* (1998) *Br J Pharmacol* 123:1331-1336).

The present invention is further illustrated by the following examples which in now way should be construed as being further limiting. The contents of all cited references a (including literature references, issued patents, published patent applications and co-pending applications) cited throughout this application are hereby expressly incorporated by reference in their entirety.

Examples

Example 1: Methods and Materials

(A) Vector construction

A full length mouse NMDAR1 cDNA was subcloned into the AAV plasmid from the parent plasmid, pSub201 under the control of a CMV immediate-early promoter and bovine growth hormone (bGH) polyadenylation site between the AAV inverted terminal repeats, as previously described (During *et al.* (1998) *Nature Med.* 4:1131 - 1135). Recombinant AAVNMDAR1 virus was generated using the helper-free packaging system as described by During *et al.* (1998), *supra*. Alternatively, the full length mouse cDNA can be subcloned into the AAV plasmid in a similar manner. The detailed discussion that follows is to the NMDAR1 receptor, although comparative results can be obtained with the Glu R receptor.

(B) Peroral administration

Male Wistar rats (300-350g) were obtained from the Animal Resources Unit, Univ. of Auckland and studies approved by the Animal Ethics Committee. Rats were fasted overnight with access to water only before being anaesthetized with ketamine/xylazine (60 mg/6 mg; per kg i.p.) and vector (AAVNMDAR1 or control AAVlac) diluted in PBS to 1 x 10^9 infectious units in a final volume of 120 μl, was administered via an orogastric tube. Rats were allowed to recover and were returned to standard rat chow 20 h after vector administration.

(C) PCR Amplification and Genomic DNA

Eight months after viral administration, animals were sacrificed and genomic DNA was extracted from gut, testes, spleen and liver using standard methods. 200 ng DNA, 400 nM of CMV primers, CMV-1 (5' CCCAGTACATGACCTTATGGG 3') (SEQ ID NO: 1) and CMV-2 (5' GGAGACTTGGAAATCCCCGT 3') (SEQ ID NO: 2) were used in conjunction with the PCR master kit (Boehringer Mannheim) to amplify a 141 bp product. The cycling parameters were 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 30 sec at 49°C, 30 sec at 72°C. Analysis of β-actin genomic DNA was used to monitor DNA integrity. β-actin primers β-A1 (5' CTCTTCCAGCCTTCCTTCC 3') (SEQ ID NO: 3) and β-A2 (5' GTCACCTTCACCGTTCCAG 3') (SEQ ID NO: 4) were used to amplify a 772 bp band. After amplification, 5ml of PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized under UV.

(D) IgG isolation and purification

Total IgG from AAVNMDAR1, AAVlac and naïve serum was isolated using an ImmunoPure (G) IgG Purification Kit (Pierce, Rockford, EL). After acid elution, the IgG fractions were neutralized and dialysed against PBS. SDS-PAGE analysis confirmed purity of the sample. Any contaminating albumin was removed using affigel blue Biorad). The final purity of each IgG sample was >98%. To ensure the immunoreactive species had been successfully isolated, each AAVNMDAR1 IgG preparation was screened for anti-NMDAR1 activity.

(E) Primary neuronal cultures

Pregnant Wistar rats were overdosed with pentobarbital under aspectic conditions, and the midbrain region was dissected from E15 embryos into warm dissecting medium (Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution containing 0.6% glucose, 100 U/ml penicillin, 100 mg/ml streptomycin, 15 mM HEPES; Gibco BRL). Tissue was digested in 0.25% trypsin (Gibco BRL) containing 200 μg/ml DNase (Sigma) in dissecting medium for 15 min at 37°C in a shaking waterbath. Trypsin digestion was terminated by the addition of trypsin inhibition medium (100 μg/ml soybean trypsin inhibitor (Sigma), 20% FCS (Gibco BRL), 200 μg/ml DNase in dissecting medium), and the tissue washed twice in dissecting medium containing 10% FCS and 200 μg/ml DNase. A cell suspension was obtained by tissue trituration and filtration through a 100 γm nylon filter. Cells were pelleted at 400 g, resuspended and plated onto poly-l-lysine-coated coverslips at a density of 250,000 cells/cm^2 in Neurobasal™ medium containing B27 supplement and 0.5 mM L-glutamine (all from Gibco BRL). Medium was replenished every 48 h, with the addition of a mitotic inhibitor (0.5 μM cytosine arabinoside) after 4 days. Cultures were grown for at least 9 days prior to calcium imaging.

(F) MTT assay

Cells were plated out in 96-well plates as above. Antibody-treated cells were incubated in purified IgG fraction for 16 hours prior to addition of NMDA medium for a further 22 hours before addition of 20 ml of 5 mg/ml MTT (Sigma M2128) in PBS to each well for 2 hours at which time purple crystals were readily apparent in the cells. 10% SDS in 0.01 M HC1 was added and left overnight to break up the cells. Absorbance was then read at 595 nm on an ELISA plate reader.

(G) Immunohistochemistry and autoradiography

Four weeks or 5 months after vector administration, rats were overdosed with pentobarbital. The gastrointestinal tract was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight, followed by cryoprotection in 30% sucrose in PBS. For the gut cell marker immunohistochemistry, representative portions of the gut were freshly frozen. Twenty micron sections from the proximal intestine were cut on a cryostat and thaw mounted onto poly-lysine coated slides. Immunohistochemistry using a monoclonal NMDAR1 antibody (1:250 dilution, Chemicon) and propidium iodide staining was conducted as described previously (During *et al.* (1998) *Nature Med.* 4: 1131).

For gut cell markers, double label immunohistochemistry was performed using monoclonal antibodies to SIRP (1:50, Chemicon) and dendritic cells (1:5, Chemicon), followed by a unlabelled secondary anti-mouse IgG blocking step prior to NMDAR1 immunohistochemistry, with detection using secondary anti-mouse antibodies conjugated with Cy3 or Cy5 (1:250, Jackson Immunoresearch). Immediately prior to confocal imaging, sections were incubated with acridine orange (0.01% in PBS, pH 6.2) for 1 min, washed in PBS and mounted in Vectashield (Vector).

Brain immunohistochemistry was performed as described previously on slide-mounted sections (Young *et al.* (1999), *Nature Med.* 5: 448). Following fixation and washing, sections were incubated overnight at room temperature with a rabbit antibody to clusterin (1:200 provided by D. Christie, University of Auckland) diluted in PBS-Triton containing 1% normal goat serum and 0.4 mg/ml thiomersal. Sections were washed before application of secondary anti-rabbit FITC (Jackson Immunoresearch) followed by NeuN (1:1000, Chemicon) immunohistochemistry and detection with a secondary anti-mouse Cy3 conjugated antibody (Jackson Immunoresearch). Immunofluorescent signals were captured using a Leica 4d TCS confocal microscope and all images processed using Adobe Photoshop 4.0 (Adobe Systems). Immunohistochemistry with a commercial NMDAR1 antibody (Chemicon) and naïve, AAVlac or AAVNMDAR1 purified IgG at a concentration of 50 μg/ml was conducted on sections from naïve rats that had been perfused with PBS. Sections were washed before application of biotinylated secondary goat anti-rabbit or rat antibodies (1:250; Sigma) followed by ExtrAvidin peroxidase (1:250; Sigma) incubation and DAB visualisation (young *et al* (1999) supra). Peroxidase-conjugated Isolectin-B4 (1:50, Sigma) was applied to fixed sections followed by DAB visualisation.

(H) TUNEL staining

TUNEL staining was performed prior to NeuN immunohistochemistry on 16 μm frozen coronal hippocampal brain sections as described previously (*44*) with the following modifications. After fixation and washing, sections were preincubated with 100 μl TdT buffer (Gibco BRL) for 10 min at RT followed by a 1 h incubation in a reaction mixture containing 0.75 μl biotin 14-dATP (Gibco BRL), 0.75 μl TdT (Gibco BRL), 15 μl 5xTdT buffer and 58.5 μl distilled water at 37°C. After the 2xSSC rinse and incubation with 2% BSA in PBS-Triton, sections were incubated with 200 μl ExtrAvidin-FITC (1:100, Sigma) for 1 h. Negative and positive controls were conducted as described previously by Young *et al* (1999) *supra*.

(I) Calcium imaging

Primary mescephalic neurons were incubated for 16 h with the purified IgG at a concentration of 50 μg/ml prior to loading with 2 μM Oregon Green 488 BAPTA-1 (Molecular Probes) for 30 min followed by a HEPES-buffered saline washes. Confocal images were collected using a Leica 4d TCS confocal microscope at 4 sec intervals before cells were challenged with 100 pM NMDA (in Mg^{2+}-free solution containing 3 μM glycine). The images were pseudocoloured according to fluorescent intensity, with red representing basal Ca^{2+} levels and yellow representing higher Ca^{2+} concentrations. Changes in fluorescent signal were assessed by measuring fluorescent intensity in cells relative to basal levels using NIH image (NIM and expressed as a ratio of fluorescent intensity following NMDA challenge over basal levels.

(J) Immunoblots

For ß-galactosidase antibody screening, 1 μg purified ß-galactosidase protein (Sigma) was separated on a 10% acrylamide gel under reducing conditions and transferred to a nitrocellulose membrane. Serum samples from AAVNMDAR1, naïve and AAVlac animals (1:200), or monoclonal ß-galactosidase (1:5000, Gibco BRL) were applied for 1 h at room temperature (RT) or overnight at 4°C following a 90 min incubation in Tris-buffered saline containing 0.1% Tween 20 (TBST) containing 5% fetal calf serum (FCS) to block non-specific binding. Bound antibodies were detected using a peroxidase-labeled anti-rat or mouse antibody (1:12,000, Sigma) for 1 h at RT, and visualized using the ECL detection system (Amersham). Hippocampal and cortical extracts were prepared from naïve rat brain. Two preparations were used: (i) a crude hippocampal or cortex extract was prepared by homogenizing the tissue in ice cold 320 mM sucrose in 10 mM Tris-HCl, pH 7.4; (ii) a non-denatured membrane extract was prepared by homogenizing tissue as described above, in the presence of protease inhibitors (Mini Complete™, Boehringer Mannheim). Following centrifugation at 7000g, 10 min, 4°C, the resulting supernatant was centrifuged at 37,000 g, 40 min, 4°C and the pellet resuspended in 10 mM Tris-HCl, pH 7.4 containing protease inhibitors. For NMDAR1 antibody screening, 20 μg total hippocampal extract was separated on a 12% reducing gel or 20 μg non-denatured hippocampal membrane protein on a 10% reducing gel, transferred to nitrocellulose, and blocked. As above, serum (1:200) was screened along with a monoclonal NMDAR1 (1:3000, Chemicon MAB363) and/or a polyclonal NMDAR1 (1:250, Chemicon AB1516) antibody, and the bound antibodies detected as described above. AAVGAD65 serum (1:200) and commercial GAD65 antibody (Chemicon) was screened against 20mg of a hippocampal cytosolic fraction, with detection as above. Naïve, AAVlac or AAVNMDAR1 CSF (1:5 dilution) was screened against 30μg of a hippocampal extract and the ECL plus (Amersham) detection system used.

(K) Peptide preparation and epitope mapping.

Ninety-four sequential biotinylated peptides overlapping by 6 residues were synthesised (Chiron Technologies, Australia). 1.2 mmol of each peptide was reconstituted in 0.2 ml DMSO and stored as stock solutions at -20°C. Prior to use, the peptides were diluted 1:1000 in phosphate-buffered saline containing 0.1% Tween 20 (PBST). Plates were coated with streptavidin (5 mg/ml) at 37°C overnight and then blocked for 2 h at RT with 1% FCS/PBST. Diluted peptides were added and incubated for 2 h at RT. Serum from AAVNMDAR1, AAVlac and naïve rats was then added (diluted 1:200) and incubated at 4°C overnight. To detect bound IgG, peroxidase-conjugated anti-rat secondary antibody (1:40,000) was added, and after 1 h at RT, OPD substrate (Sigma) was applied and absorption at 490 nm determined. To determine the specificity of the NMDAR1 sera for each peptide, the ratio between the AAVNMDAR1 signal (absorbance at 490 nm; peptide ELISA) and mean AAVlac signals was calculated. The final values were plotted to produce the epitope maps shown in Fig. 3D-H.

(L) Kainic acid administration

Three weeks following vector, animals were anaesthetised with 60 mg/kg i.p. pentobarbital and implanted with bipolar recording electrodes (MS33-2B, Plastics One Inc.) into the right dorsal hippocampus under stereotaxic guidance ((anterior posterior (AP) -3.3 mm, medial-lateral (ML) 2.0 mm, dorsal-ventral (DV) 3.6 mm, bregma = 0). One week later, rats received 10 mg/kg kainate i.p. and seizures were monitored on a Grass 79E EEG recorder. After 45 min of seizure activity, animals were administered an anticonvulsant dose of pentobarbital (30 mg/kg i.p.). Animals were sacrificed 3 days after kainate injection. Following pentobarbital anaesthesia, brains were removed and snap-frozen on dry ice. Coronal hippocampal sections (16 μm) were taken for TUNEL and clusterin analysis.

(M) Middle cerebral artery occlusion (MCAO)

Five months after vector administration, endothelin-1 (60 pmol in 3 μl saline; Novabiochem) was injected via a 30 g cannula above the middle cerebral artery (AP +0.2 mm, ML 5.9 mm, DV from dura 7.5 mm) of anaesthetised animals (Darnell (1996) *Proc Natl Acad Sci USA* 93: 4529). Following recovery from surgery, animals were sacrificed 3 days later. Brains were removed, frozen and 20 μm coronal sections taken for haematoxylin-eosin staining and isolectin B4 analysis. Infarct volume estimates were determined on serial haematoxylin-eosin stained sections using the Cavalieri method (Gundersen *et al.* (1988) *APMIS* 96: 857).

(N) IgG penetration

To investigate basal and kainate-induced IgG penetration, vaccinated animals (naïve n=4, AAVlac n=4, AAVNMDAR1 n=4) and AAVNMDAR1 animals 90 min after kainate administration (Young *et al.* (1999) *Nat. Med.* 5: 448-453) (n=2) were anaesthetised before perfusion with PBS to remove endogenous IgG present in blood vessels. The brains were removed and frozen on dry ice before 16 μm coronal sections at the level of the hippocampus were taken for anti-rat IgG (1:250, Sigma) immunohistochemistry.

(O) ^{3}H-MK-801 autoradiography

Hippocampal sections were incubated in 50 mM Tris HC1 containing 2.5 mM $CaCl_2$, pH 7.4 for 1 h and dried before being incubated with 20 nM ^{3}H-MK-801 (NEN) for 1 h. Non-specific binding was determined by labelling in the presence of 100 μM cold MK-801. Sections were rinsed twice in ice-cold Tris HCI buffer for 1 min each, followed by a quick dip in ice-cold distilled water and were dried overnight at 4°C before being exposed against ^{3}H-Hyperfilm (Amersham) for 3 weeks. Density measurements were made from autoradiograms using NIH image. Statistical analysis was conducted using Student's t-test.

(P) In situ hybridization

In situ hybridization was performed on 16 Pin frozen hippocampal sections from AAVlac and AAVNMDAR1-vaccinated animals using oligonucleotide probes to NMDAR1 (5'CAC AGC CTG GAT GGC CTC AGC TGC GCT CTC GTA ATT GTG TTT T 3') (SEQ ID NO: 5), NMDAR2A (5'AGA AGG CCC GTG GGA GCT TTC CCT TTG GCT AAG TTT C 3') (SEQ ID NO: 6), NMDAR213 (5'CAT GTT CTT GGC CGT GCG GAG CAA GCG TAG GAT GTT GGA GTG GGT 3') (SEQ ID NO: 7) and trkB as described previously (Young *et al.* (1999) *Nat. Med.* 5: 448-453). Density measurements were conducted on film autoradiograms as described above for H-MK-801 binding.

(Q) Cerebrospinal fluid (CSF) sampling

A subgroup of animals were anaesthetised with 60 mg/kg i.p. pentobarbital and a non-traumatic sample of CSF (80-100 μl) was obtained from the cisterna magna using a 27 g needle. Rats were left at least 7 days following CSF sampling before kainate injection (10mg/kg i.p). Ninety min later, rats were anaesthetised as described above and CSF sampled.

(R) Behavioural tests

(i) Barnes Circular Maze - This was carried out as described previously (Barnes *et al.* (1979) *J. Comp. Physiol. Psychol.* 93: 74-104). Briefly, rats use spatial navigation to escape from a brightly-lit elevated circular 2 m diameter table which has 18 equally spaced holes around the circumference, one hole which leads to an escape box. On the first day of testing, each rat was placed in the escape box for a four min adaptation period. After one min in the home cage, trial 1 began. On subsequent days, two trials were conducted, separated by one min in the home cage. Testing continued for six days, (11 trials in all). For each trial, rats were placed in the centre of the table under a cylindrical start box for 30 sec, then allowed four min to find and enter the escape tunnel. During this time, the number of incorrect holes searched and latency to enter the tunnel were recorded. All animals spent one min in the tunnel at the conclusion of their trials. Between trials, the table was cleaned with 70% ethanol, and the hole under which the tunnel was placed, though always in the same spatial location, was randomly determined for each rat. From trial 8 onwards, the position of the escape box was altered by 135 degrees, to control for the possibility that rats had learnt to navigate to the escape box by other than spatial means.

(ii) Line crossing mobility test - A 2 meter diameter circular table was divided into 9 segments of approximately equal size. Each rat was placed in the centre of the table, and allowed 5 min of free movement during which a record was made of the number of times the rat's two front feet crossed a line separating two segments. Testing was conducted for 5 days, (1 trial per day). Between trials the table surface was cleaned with 70% ethanol.

(iii) Circular track mobility test - The track used was a modified version of one used to test mobility in mice. Each rat was placed inside the track at the start position, facing clockwise, and the number of circuits completed in 5 min was recorded. This procedure was conducted for 5 days.

(iv) Contextual Fear Conditioning - Each rat was placed in a metal operant chamber (Med Associates Inc.) for 2.5 min of exploration. A tone was then sounded for 30 sec, with a 0.4 mA shock administered during the last 2 sec. 1 h later, rats were returned to the chamber, and scored for the number of 5 sec intervals spent frozen over a 5 min period. Results were analyzed with Systat v5.2. (Systat). Two way ANOVA tests were used, with rat type as the explanatory variable, and day and time (first or second trial of day) as repeated measures where appropriate. Individual trials were analyzed using a Wilcoxon Rank Sum or two-tailed independent t-test.

(S) Nociception Pain Test

The hot plate and tail-immersion tests were performed to assess the effects of vaccinations on nociception. The hot plate test was performed by placing the rat on an aluminum plate maintained at 55°C (Barnstead Thermolyne Co.) and measuring the latency in detecting a nociceptive response identified as a licking of a hindpaw or an escape response (jumping over a 20 cm barrier). Any rat that did not respond within 15 sec would have been removed from the hot plate, but all rats responded within 10 sec. The tail immersion test was performed by wrapping the rat in a towel then immersing the tail in a beaker of water kept at a constant 55°C temperature. The latency for the animal to either remove the tail or elicit a jerk response was measured. Any rat not responding within 15 sec would immediately be removed from the water, however all tested rats responded within 10 sec. All behavioural tests were carried out by blinded investigators.

Example 2: Immunization of Animals with the AAVNMDR1 Genetic Vaccine.

A full length mouse NMDAR1 cDNA was subcloned into an adeno-associated virus (AAV) plasmid and subsequently packaged and purified to yield a high titre recombinant AAV virus, AAVNMDAR1 (Fig. 1A and Fig. 1C). Fig. 1A shows the plasmid map of the NMDAR1 construct. Fig. 1C shows transduction of HEK 293 cells (arrows) by AAVNMDAR1 using NMDAR1 immunocytochemistry. This vector was administered via an orogastric tube into the stomach of a group of rats with a control group receiving a similar dose of a recombinant AAV virus expressing beta-galactosidase (AAVlac) (During *et al.* (1998) *Nature Med.* 4:1131 - 1135).

(i) Oral genetic vaccination with AAVNMDAR1

To demonstrate successful peroral administration of the vaccine and production of antibodies, genomic DNA and protein expression in the intestine was examined. Oral vaccines are usually scavenged by intestinal M cells, rapidly taken up by the antigen presenting cells (APC) in Peyers patches and the lamina propria, and can induce strong humoral immune responses (Shalby *et al.* (1995) *Clin. Immunol. Immunopath* 74:127).

Fig. 1B, is a photograph of an agarose gel showing PCR amplification of CMV promoter from genomic DNA extracted from AAVNMDAR1 and AAVlac-vaccinated rats. (Lane 1, kb plus DNA ladder; Lane 2, DNA extracted from the gut of an AAVNMDAR1 rat; Lane 3, DNA extracted from the gut of an AAVlac rat; Lane 4, DNA extracted from control naïve rat gut; Lane 5,6,7, show DNA extracted from the liver, spleen and testes respectively, of a AAVNMDAR1 rat; Lane 8, no template control; Lane 9, positive control (CMV promoter amplified from AAVNMDAR1 plasmid. Amplification of CMV with this set of primers generated a 141 bp product consistent with that found for the positive control and Lanes 2 and 3 (lower arrow). b-actin genomic DNA served as a control of DNA integrity (upper arrowhead).

The results show that in rats treated with the oral AAVNMDAR1 vector, transduction of intestinal cells was confirmed by PCR amplification of a portion of the CMV promoter from genomic DNA isolated from the proximal intestine. Similarly, the PCR product was also detected in AAVlac-immunized rat intestine, but not detected in DNA from the intestine of naïve rats, or in the liver, spleen or testis of any AAVNMDAR1- or AAVlac-immunized rats (Fig. 1B).

(ii) Protein Expression

The expression of NMDAR1 was determined. NMDAR1 protein expression in the intestine was detected at four weeks (Fig. 1D-F) and five months (Fig. 1H) following peroral AAVNMDAR1 administration. Double immunofluorescence analysis with propidium iodide was used to show the lamina propria (lp) and epithelial (ep) cell layers. NMDAR1 immunohistochemistry showed NMDAR1 protein expressed within these two regions. NMDARI Protein expression was also determined using a commercially available NMDAR1 primary antibody and fluorescent secondary antibody as described in Example 1. NMDAR1 protein was not expressed in AAVlac-treated animals at 4 weeks (Fig. 1G) or 5 months (not shown).

Using confocal microscopy imaging, expression was observed primarily in the lamina propria as previously described for AAVlac-treated animals (During *et al.* (1998) *Nature Med.* 4: 1131). At a dose of 10^9 rAAV infectious particles, a total of 9-12 million lamina propria cells were transduced at 4 weeks with approximately ~10 million immunoreactive cells remaining at 5 months with no loss of expression, as previously reported using *E.coli* lacZ as the transgene (During *et al*, *supra*). Double label immunofluorescent staining combined with acridine orange counterstaining (green) to visualise nuclei showed colocalisation of NMDAR1 protein in Fig. 2B and Fig. 2D with antibodies to gut cell markers (Fig. 2A) SIRP and (Fig. 2C) dendritic cells. These results showed that transduced cells were immunoreactive using the antibodies MRC OX-41 and OX-62 to define signal regulatory proteins expressed on cells of myeloid origin (SIRP) and dendritic cells respectively confirming the ectopic NMDAR1 expression in professional antigen-presenting cells.

(iii) Circulating antibodies

To determine the presence of circulating NMDAR1 antibodies, blood was removed from the vaccinated animals and the serum analyzed for presence of specific antibodies. Fig. 3A shows the results from sera (1:200) from AAVlac, AAVNMDAR1 and naïve control rats, screened by immunoblot analysis for the presence of β-galactosidase antibodies. 1 μg purified β-galactosidase was loaded per lane. (Lane c, commercial β-galactosidase antibody; Lane 1, naïve control serum; Lane 2, AAVNMDAR1 serum 4 weeks after vaccination; Lane 3, AAVlac serum 4 weeks after vector administration; Lanes 4-5, AAVlac serum from two individual animals 4 months after vector). The results show that in AAVlac-treated animals, IgG antibodies were detected at 4 weeks with titres further increasing at 16 weeks. Using a purified beta-galactosidase enzyme preparation, a commercial monoclonal antibody recognized two molecular weight protein species of 116 and 85kD on immunoblots (Fig. 3A). Some animals had antibodies that bound preferentially to the 85kD species whereas other animals had serum antibodies with higher affinity to the 116kD protein (Fig. 3A).

Fig. 3B shows the results from serum (1:200) from AAVlac, AAVNMDAR1 and naïve control rats, screened by immunoblot analysis for the presence of NMDAR1 antibodies. 20 μg hippocampal membrane extract was loaded per lane. (Lane c, commercial NMDAR1 antibody (Chemicon, MAB363); Lane 1, naïve serum; Lane 2, AAVlac serum (4 weeks); Lane 3, AAVNMDAR1 serum (4 weeks); Lane 4, AAVNMDAR1 serum (4 months)). The results showed that immunoblotting of AAVNMDAR1 serum to a hippocampal membrane extract yielded the expected 117kD band, consistent with the molecular weight of the native NMDAR1 receptor subunit and similarly recognized by commercial antibodies. The sera from naïve control (n=4) or AAVlac (n=12) immunized animals showed no binding to the brain extract.

Fig. 3C shows the results from sera (1:200) screened against a denatured hippocampal extract, 20 μg was loaded per lane. (Lane P, polyclonal NMDAR1 antibody (Chemicon AB1516); Lane M, monoclonal NMDAR1 antibody (Chemicon, MAB363); Lanes N35, N64, N52, is AAVNMDAR1 serum from three different animals showing specific affinities for different NMDAR1 breakdown products.) Brain extracts prepared under more severe denaturing conditions resulted in degradation of the native receptor as shown by detection with a commercial monoclonal antibody which bound fragments running at 32kD and 67kD. Of interest, individual AAVNMDAR1 rats showed different patterns of binding to some of these fragments (Fig. 3C Lanes N35, N64 and N52). These data demonstrate that AAV can serve as an oral vaccine and that immunized animals generated antibodies against a range of NMDAR1 epitopes.

(iv) Epitope mapping of NMDAR1

To further define the range of epitopes, a total of 94 overlapping 16mers were synthesized covering the entire 938 amino acids of the native NMDAR1 protein as described in Example 1K. Serum from AAVNMDAR1, AAVlac and naïve rats were screened against this panel of 16mers.

Figs. 3D-H of rats N11, N19, N21, N52, N64 show the epitope map profiles of five different AAVNMDAR1-treated animals. Specificity is measured as a ratio between the AAVNMDAR1 signal and mean AAVlac signals for each peptide. The results showed that none of the naïve (n=4) or AAVlac rats (n=14) screened had specific binding to any of the peptides. In contrast, AAVNMDAR1-immunized rats showed specific binding to peptides which corresponded to functional domains within the extracellular segments of the receptor. These included peptide 49 (amino acids 483-498) which represented the N-terminal side of M1, the first transmembrane domain (rat N19, Fig. 3E), and two peptides corresponding to the extracellular domain between M3 and M4, peptide 69 (amino acids 681-696; rat N21, Fig. 3F) and peptide 72 (amino acids 711-726; rat N64, Fig. 3H). Each of these three peptides contain critical residues for glycine binding (Kuryatov *et al* (1994) *Neuron* 12: 1291; Wafford *et al.* (1995) *Mol. Pharmacol.* 47: 374; Wood *et al.* (1997) *J. Biol. Chem.* 272: 3532). The most common pattern observed in serum from 7 of the 19 AAVNMDAR1 rats screened was specific binding to peptides adjacent to the M4n region (peptide 80, amino acids 791-807) and/or peptide 65 (amino acids 641-657) corresponding to the M3c domain (rat N11, Fig. 3D). Two additional rats had antibodies that bound to peptides 54/55 (amino acids 541-566; rat N52, Fig. 3G) which mapped to the preM1 domain. The preM1, M4n and M3c regions form part of the extracellular vestibule of the NMDA receptor channel where amino acid substitutions at key residues have a significant influence on channel permeability (Beck *et al.* (1999) *Neuron* 22: 559).

The AAVNMDAR1 vaccinated rats generated autoantibodies which were polyclonal. Epitope mapping of the antibodies showed a diverse range of epitopes in multiple regions of the extracellular domains. Individual animals had antibodies which bound to 16mers that mapped to the extracellular vestibule of the channel including the preM1, M3c, M4n domains (Beck *et al.* (1999). *Neuron* 22: 559), as well as epitopes in the N terminal region and within the extracellular loop lying between M3 and M4 which directly mapped to glycine binding sites (Kuryatov *et al.* (1994) *Neuron* 12, 1291; Wafford *et al.* (1995) *Mol. Pharmacol.* 47, 374; Wood, *et al.* (1997) *J. Biol. Chem.* 272, 3532). Several animals did not appear to have an immunodominant epitope using the set of overlapping 16mers, a result consistent with antibodies which are dependent on the conformational state of the protein.

In summary, rats were immunized against a specific brain protein, the NMDAR1 subunit of the NMDA receptor, and autoantibodies to the receptor were generated. These antibodies were able to bind to the NMDA receptor demonstrating specific targeting of a functional domain of the protein. The epitope-mapping analysis demonstrated that the autoantibodies bound to known functional regions of the protein including the extracellular vestibule of the channel as well as to peptides which contained glycine binding sites.

Example 3: Neuroprotection against epilepsy using the NMDAR1 genetic vaccine.

To assess the anti-epileptic and neuroprotective efficacy of AAVNMDAR1 vaccination, the kainate model of temporal lobe epilepsy was used as described in Example IL. The systemic administration of kainate is a well established model of temporal lobe epilepsy (*See* Sperk (1994) *Prog. Neurobiol.* 42: 1). Animals received AAVNMDAR1 (n=9) or AAVlac (n=8) and a group of naïve control rats received no vector (n=17). At one month following vaccination, animals were administered 10 mg/kg kainic acid intraperitoneally and a blinded observer determined over 2 hours whether there were any signs of the characteristic progression through various behavioural seizure stages, including immobility and staring, 'wet-dog-shakes', facial clonus, unilateral and bilateral forelimb clonus (Sperk (1994) *Prog. Neurobiol.* 42:1)

Fig. 4A shows that kainate-induced seizure activity was evident shortly after drug administration as shown by EEG recordings. The first signs of electrographic seizure activity was observed within 10 minutes following drug administration in control animals, with 13 out of 17 naïve and 6 of the 8 AAVlac animals developing facial and forearm clonus and proceeding to status epilepticus (SE). In contrast, only 2 out of the 9 AAVNMDAR1-vaccinated animals developed seizures and SE (Table 1). The remaining 7 AAVNMDAR1 rats showed neither EEG changes nor any behavioural changes following kainate (p=0.007, Chi Square analysis with expected SE frequency of 68% reduced to 22% in the NMDAR1-immunized group). In those rats which exhibited SE, seizures were terminated with an anticonvulsant dose of sodium pentobarbital (30 mg/kg i.p.) after 45 minutes. Animals that did not develop seizures also received pentobarbital (30 mg/kg i.p.)

To confirm that the AAVNMDAR1 vaccination was able to suppress kainate-induced seizures, the hippocampi of all animals was examined for any signs of seizure-induced neuropathology. Three days after kainate treatment, animals were euthanised and then brains were removed and frozen and coronal hippocampal sections were prepared for immunohistochemistry and analysed using TUNEL and clusterin analysis, as described in Example 1G and H.

Figs. 4B, D, F, H, and J show the analysis of hippocampal damage using fluorescent TUNEL labelling, while Figs 4C, E, G, I and K show the analysis of hippocampal damage using clusterin immunohistochemistry combined with immunohistochemistry with NeuN, a mature neuronal marker. As shown in the hilar region, all AAVlac animals (Figs. 4B,C) that developed SE showed numerous TUNEL-positive (arrows) and clusterin-immunofluorescent neurons, indicative of extensive neuronal death in the hippocampus. No TUNEL or clusterin immunofluorescence was found in any AAVNMDAR1-vaccinated rat that did not have seizures (*See* Figs. 4D,4E).

One AAVNMDAR1-vaccinated rat that developed SE also showed extensive clusterin (Fig. 4G) and TUNEL staining (Fig. 4F). Of interest, the second animal that developed SE showed no TUNEL signal (Fig. 4H) or clusterin immunofluorescence (Fig. 4I). The EEG recordings shown in (Fig. 4A) correspond to the brains shown in (Fig. 4B-4I). Kainate-induced seizures were also elicited in AAVGAD65-vaccinated animals and TUNEL (Fig. 4J) and clusterin labelling (Fig. 4K) confirmed extensive neuronal damage in the hippocampus. Scale 200 μm.

The results demonstrated that no discernible TUNEL signal or clusterin immunofluorescence (a cell death marker, *see* Dragunow *et al.* (1995) *Mol. Brain Res.* 32: 279) was observed in the hippocampus of any animal (AAVlac, naïve or AAVNMDAR1) that did not develop SE (Fig. 4D,E). In comparison, all AAVlac and naïve animals that experienced SE had numerous TUNEL-positive and clusterin-immunofluorescent neurons in hippocampal CA1, CA3 and hilar regions (Fig. 4B,C). Of the two AAVNMDAR1 animals that had SE, only one of these animals (rat N12) had some injury (Fig. 4F,G), whereas the second animal (N7) had no injury whatsoever, despite over 45 minutes of severe SE (Fig. 4H, I, Table 1).

As seizure duration is a critical determinant of the extent of neuronal injury, with as little as 30 minutes duration being sufficient to cause cell death (Lothman *et al.* (1993) *Epilepsia* 34: S59), these results suggest that the AAVNMDAR1 vaccination can not only produce resistance to seizures but also protect against excitotoxic injury. Of interest, N12, the only rat to have SE as well as brain injury following AAVNMDAR1 oral dosing, was also the only animal of the 9 in this experiment that did not have detectable circulating antibodies to the NMDAR1 receptor subunit.

(i) NMDA Receptor Specificity

To determine whether the anti-epileptic and neuroprotective effect of the NMDAR1 vaccination was non-specific and could be reproduced with immunization against any brain protein, an AAV vector expressing glutamic acid decarboxylase-65

(GAD-65) was generated. The vector expressing GAD-65 was perorally administered to an additional group of rats. These rats all developed significant antibody titres to the native protein. At one month following vaccination, these rats were challenged with kainate as described above. All of these rats (n=6) rapidly progressed to motor seizures and SE (Table 1). Analysis of their brains showed extensive damage (Fig. 4J,K confirming severe seizure activity. These results demonstrate the specific involvement of NMDA receptors in epilepsy.

Table 1 Summary data showing the neuroprotective effect on epilepsy in rats vaccinated with the AAVNMDAR1 vaccine

	Number developing SE	Hippocampal injury in SE rats		
		-	++	+++
Naïve	13 (17)	-	-	13
AAVlac	6 (8)	-	-	5
AAVNMDAR1	2 (9)	1	1	-
AAVGAD-65	6 (6)	-	-	6

Hippocampal injury in rats that had developed SE was graded according to the extent of injury, - no injury, ++ moderate (<20 TUNEL or clusterin-immunofluorescent cells), +++ extensive (>20 TUNEL or clusterin-immunofluorescent cells). Numbers in parentheses represent total number of rats in that group. No injury was found in rats that did not develop SE.

(ii) Antibody passage across an intact blood-brain barrier

To confirm passage of NMDAR1antibodies into the brain, at 4 weeks a group of vaccinated rats (n=4) underwent non-traumatic sampling of cerebrospinal fluid (CSF) from the cisterna magna. Fig. 5A shows the results of an immunoblot analysis of AAVlac, AAVNMDAR1 serum and CSF and AAVGAD serum. 30 micrograms of hippocampal membrane extract was separated on a reducing gel under non-denaturing conditions. Sera (1:5000) from AAVlac, AAVNMDAR1, AAVGAD and naïve rats were screened for the presence of NMDAR1 antibodies; (lane 1, polyclonal NMDAR1 antibody (Chemicon AB1516, 1:5000); lane 2, naïve sera; lane 3, AAVlac sera; lane 4, AAVNMDAR1 sera; lane 5, AAVGAD sera). CSF (1:5) from the same animals was also screened for NMDAR1 antibodies; (lane 6, naïve CSF; lane 7, AAVlac CSF; lane 8, AAVNMDAR1 CSF under basal conditions; lane 9, CSF from the same rat sampled 2h after kainate administration). Serum from AAVGAD-65-vaccinated rats was screened for the presence of GAD65 antibodies against a hippocampal extract using immunoblot analysis. (Lane 1, commercial GAD65 antibody, Lane 2, 3 serum from two individual animals 1 month after vaccination).

Figs. 5B-D show the results of anti-rat IgG immunohistochemistry showing IgG penetration was significantly enhanced in the hippocampus following kainate treatment in the hilus and CA2-CA3 region (Fig. 5B, arrows). Fig. 5C is a high powered image of the CA3 region in (Fig. 5B) compared to the same region under basal conditions (arrows, Fig. 5D). Immunohistochemistry was conducted on control hippocampal sections using IgG purified from AAVNMDAR1 (Figs 5E, I), AAVlac (Figs. 5F,H) or naïve rat serum (not shown). As shown in the CA3 (Figs. 5E-G) and hilar region (Figs. 5H-J), only AAVNMDAR1 purified IgG showed a selective immunoreactive staining pattern which was similar to that found with a commercial polyclonal NMDAR1 antibody (Figs. 5G,J), while both naïve and AAVlac IgG (Figs. 5F,H) produced only low level background staining Scale in Fig. 5B was 500 μm, in Figs. 5C, D, E-F, was200 μm, and in Fig. 5H-J was 80 μm.

Using immunoblot analysis and a highly sensitive chemiluminescent detection method, NMDAR1 autoantibodies detected in the serum of AAVNMDAR1-vaccinated rats were also detected at low levels in the CSF by immunoblotting, demonstrating autoantibody passage into the brain (Fig. 5A). NMDAR1 autoantibodies were not detected in CSF from naïve (n=4) or AAVlac (n=4) immunised rats. Following kainate treatment, an approximate 10-fold increase in CSF levels of NMDAR1 autoantibodies was observed (Fig 5A). In addition, IgG immunoreactivity was specifically increased in the hippocampus in the CA3 and dentate hilar neurons which are also susceptible to kainate-induced neuronal damage but which were protected in NMDAR1-immunized rats (Fig 5B-D). Furthermore, immunohistochemistry using IgG purified from AAVNMDAR1, AAVlac or naïve serum was conducted on hippocampal sections from naïve animals that had been perfused with PBS to remove endogenous IgG present in blood vessels. Only AAVNMDAR1-purified IgG showed selective immunoreactivity in hippocampal CA3, dentate hilar and CA1 neurons consistent with the pattern of expression of NMDAR1 using a commercial polyclonal antibody (Fig 5E-J).

A splenocyte proliferation assay was also used to investigate whether was is a cellular-mediated immune component associated with the vaccine. No difference in proliferative response was found between splenocytes isolated from AAVlac and AAVNMDAR1 animals challenged with the appropriate antigen (results not shown). In addition, immunohistochemistry with CD8+ and MHC Class I antibody markers on hippocampal sections from naïve, AAVlac and AAVNMDAR1 rats showed no difference in CD8+ or MHC Class I immunoreactivity suggesting that the likely mechanism of action of the vaccination is mediated through a humoral response and is consistent with the association between antibody titre and the neuroprotective phenotype observed.

In summary, the results demonstrate that in serum antibody titres obtained from vaccinated animals, low levels of NMDAR1 antibodies were detected in the CSF under basal conditions using a highly sensitive chemiluminescence detection system. However, a substantial increase in the antibodies was observed in the CSF following insult/injury to the blood-bain barrier by kainate administration.

A major limitation of the successful translation of promising NMDA receptor antagonists to the clinic has been the significant profile of CNS adverse effects associated with these drugs (Schehr (1996) *Nat. Biotechnol.* 14, 1549). The invention provides a vaccine or antibody approach to NMDA receptor antagonism with the advantage that the receptor blockade is minimal under resting physiological conditions where high serum titres of antibodies do not pass the blood-brain barrier efficiently. However, following a neuronal insult, the blood-brain barrier has increased permeability to serum antibodies, and transport and subsequent binding to the target protein can occur. This "on demand" or selective delivery of the neuroprotective agent (the autoantibody) limited both spatially to the site of injury and to the precise timing of injury is advantageous feature of the invention.

Of particular interest, glutamate itself alters blood-brain barrier permeability (Mayhan *et al.* (1996) *Stroke* 27, 965). Hence the vaccine strategy of the present invention may induce an autoprotective loop. Without being limited to a mechanism of action, a cerebral insult may increase brain extracellular glutamate, which increases blood-brain barrier permeability locally, resulting in facilitated passage of the autoantibody and antagonism of glutamate receptors.

Example 4: Neuroprotection against stroke damage using the AAVNMDAR1 Vaccine

The endothelin-1 model of middle cerebral artery occlusion (MCAO) described by Sharkey *et al.*(1995) *J. Neurosci. Methods* 60: 125), was used to determine the anti-stroke and ischemic neuroprotection efficacy of AAVNMDAR1 vaccination. The model has been used previously to test novel anti-stroke drugs including the NMDA receptor antagonist, MK-801 (Sharkey *et al. Supra*, and Butcher *et al.* (1997) *J. Neurosci.* 17: 6939). This approach uses a fine needle to direct stereotactic delivery of endothelin-1 into the vicinity of the middle cerebral artery (MCA) and causes vasospasm leading to complete occlusion and generation of an extensive infarct in the MCA territory, including the stratium and cortex on the injected side.

Three groups of rats, untreated (n=10), AAVlac (n=8) and AAVNMDAR1 (n=10) underwent MCAO five months following vaccination. At 3 days following endothelin-1 administration, rats were euthanised, the brains removed and the infarct volume determined by a blinded investigator.

Figs. 6A-6H show the results of the study. Three days following endothelin-induced MCAO, haematoxylin-eosin staining showed infarction of the ipsilateral stratium and/or cortical regions in AAVlac-treated animals (Fig. 6B) while there was no damage on the contralateral side (Fig. 6A). High power images of the undamaged contralateral (Fig. 6C) and infarcted ipsilateral (Fig. 6D) stratium. In contrast, damage was less severe and restricted to cortical regions in the AAVNMDAR1-vaccinated animals (Fig. 6F) with no damage to the contralateral side (Fig. 6E and Fig. 6G). Increased isolectin B4 immunoreactivity in the infarcted regions suggests microglial proliferation into damaged areas (Fig. 6H). The total infarct volume was significantly less in the AAVNMDAR1-vaccinated group (n=10) compared to AAVlac-treated (n=8,) or control naïve rats (n=10). *P<0.01. Each bar represents the mean+SEM. Scale for Figs. 6A,B,E-G was 600 μm, and for Fig. 6C-D was 100 μm.

The results demonstrate that the total infarct volume was 55.6+20.2mm^3 in naïve rats and 66.4+12.4 mm^3 in the AAVlac animals, whereas in the AAVNMDAR1 rats the infarct was reduced by ~70% to 19.6+6.2 mm^3 (p=0.002, Student's t-test, Fig. 6H). When the cortex and stratium were analyzed independently, the protection in terms of infarct volume was 65% and 80% respectively compared to the AAVlac group and 69% and 74% compared to the naïve controls (Fig. 6H).

These results are of significant interest especially when considering that in partial stroke models, the most reproducible and potent data on neuroprotection has come from the use of NMDA receptor antagonists, particular the non-competitive compound, MK-801. However, even with this antagonist, at doses which depress motor activity, tissue rescue is limited to approximately 50% in the cortex with no infarct reduction in the stratium (Butcher *et al.* (1997) *J Neuorsci.* 17:6939-6946). Moreover, there is a narrow time window of only a few hours in which MK-801 and other promising new anti-stroke drugs need be given for rescue (Butcher *et al.* (1997) *J Neuorscl* 17:6939-6946) with effective protection requiring continued maintenance dosing (Steinberg *et al.* (1995) *Neuroscience* 64:99-107). Thus, the vaccination provides a neuroprotective effect against stroke damage.

Example 5: Vaccination effects on behaviour

To investigate whether the vaccination had any effect on the behaviour of the animals, the animals were tested using several established models of behaviour, (*see* Example 1R). Receptor antagonists administered systemically at anti-epileptic and neuroprotective doses typically result in some impairment in motor behaviour (*Wozniak et al.* (1990) *Pschopharmacol.* 101, 47). To determine whether vaccination was associated with any changes in motor behaviour, rats were tested on circular track and line crossing mobility paradigms

For the line crossing mobility test, a 2 meter diameter circular table was divided into 9 segments of equal size. Each rat was placed in the center of the table, and allowed 5 minutes of free movement. During this time, a record was made of the number of times the rats two front feet crossed a line separating two segments. Testing was conducted for five days, with each animal receiving one trial per day. Between trials the table was cleaned with 70% ethanol.

For the circular track mobility test, the track used was a modified version of one used to test mobility in mice (Carlsson *et al.* (1990) *Life Sci.* 47: 1729). Each rat was placed inside the track at the start position, facing clockwise, and the number of circuits completed in 5 minutes was recorded. This procedure was conducted for 5 days. Fig. 7A depicts the results from the line crossing test and Fig. 7B depicts the results from the circular track mobility test. Data represents the number of line crossings in 5 min intervals over 5 successive days in AAVlac (squares-solid line) or AAVNMDAR1 rats (diamond-dashed line). In the circular track test, the number of completed circuits in successive days for AAVlac (n=6) and AAVNMDAR1 (n=6) animals are represented.

The results from these behavioural studies did not demonstrate a difference between the groups of rats vaccinated compared with the control rats (repeated measures ANOVA, p=0.87 and p=0.32 respectively, Fig. 7A-B). The results demonstrate that in contrast to the motor impairment associated with systemic administration of most pharmacological NMDA receptor blockers, the AAVNMDAR1 vaccinated rats had no impairment in locomotor function. Accordingly, the vaccine can be used for vaccination of individuals at risk of stroke and other cerebral insults without impairment of neurological function.

Example 6: Vaccination effects on NMDA receptor-sensitive gene expression using the AAVNMDRA1 vaccine

To demonstrate that vaccination with the AAVNMDAR1 vaccine modulates gene expression, adult rats were immunized by oral gavage with recombinant AAV vectors expressing either mouse NMDAR1 (AAVNMDARl) or the *E. coli* lacZ (AAVlac) cDNAs as previously described (During *et al.* 1998, supra) (also *See*

Example 2). Serum was taken at one and four months for antibody titre and epitope mapping studies, as described in Example 1D.

(i) Modulation of NMDA receptor function

To demonstrate whether the purified IgG from the AAVNMDAR1 vaccinated rats would bind and influence the function of NMDA receptors expressed in primary neuronal cells grown in culture, primary mesencephalic rat neuronal cultures were incubated with IgG purified from AAVlac or AAVNMDARl rats. The cells were loaded with the calcium sensitive fluorescent dye, Oregon Green BAPTA-1, and the changes in fluorescent signal determined using confocal microscopy.

The results from these studies are shown in Figs. 8A-8M. Fig. 8A shows confocal images of non-treated control cells. Fig. 8D shows AAVlac IgG-treated cells, Fig. 8G shows NMDAR1 IgG-treated mesencephalic cells preloaded with the Ca^{2+} indicator Oregon Green 488 BAPTA-1 (2 μM) showed low level fluorescence. Cells were preincubated with 50 μg/ml IgG for 16 h prior to indicator loading. In response to a 100 μM NMDA+3 pM glycine challenge, the increase in fluorescent signal found in non-treated control (Fig. 8B) and AAVlac IgG-treated cells (Fig 8E) was significantly attenuated in AAVNMDAR1 IgG-treated cells (Fig. 8H). Images are pseudocoloured according to fluorescent intensity, with transition from red to yellow representing basal Ca^{2+} levels to higher Ca^{2+} concentrations. Fig. 8J shows ratio of the changes in fluorescent intensity relative to basal levels showed a significant difference between AAVlac and AAVNMDAR1 IgG-treated cells. Each bar represents the mean±SEM, $n=10$ ($*p=0.0012$, Student's t-test). Anti-rat IgG immunocytochemistry showed only purified AAVNMDARl IgG (Fig. 8I) bound to mesencephalic cells and not AAVlac IgG (Fig. 8F) which exhibited basal immunoreactivity similar to non-treated cells (Fig. 8C) Using the IgG fractions to perform immunohistochemistry on brain sections, as shown in hippocampal hilar neurons, the AAVNMDARl IgG (Fig. 8L) showed a pattern of immunoreactivity similar to that found with a commercial NMDAR1 polyclonal. antibody (Fig. 8M), while AAVlac IgG (Fig. 8K) showed only low level background immunoreactivity. Scale for Figs 8A-H was 20 μm, for Figs. 8 C, F, I, was 30 μm, and for Figs. 8K-M, was 100 μm

The results show that in untreated cells, or cells incubated with IgG from AAVlac rats (n=10) at a concentration of 50 μg/ml, a marked increase in the fluorescent signal was obtained following NNMA application (Figs. 8A,B,D,E). Mesencephalic neurons incubated with the same concentration of IgG purified from AAVNMDAR 1-immunized rats (n=10) blocked the increase in intracellular calcium (Figs. 8 G, H, J). Confirmation of specific binding was obtained by immunocytochemistry with a secondary anti-rat IgG which was applied to untreated primary mesencephalic cells or cells that had been incubated with AAVlac or AAVNMDAR1-purified IgG (Figs. 8 C,F,I). The results showed that only IgG purified from AAVNMDAR1 rat serum bound to the primary neuronal cultures (Fig. 8I). These results demonstrate that the antibodies can directly modulate the function of the NMDA receptors.

<u>(ii)</u> <u>Self-Antigen Recognition</u>

To demonstrate the recognition of self-antigens by the serum of AAVNMDAR1-vaccinated rats, immunoglobulins were purified using protein G columns and used as the source of primary antibody for immunohistochemistry of rat brain sections (*see* Example 1D and 1G). Sections at the level of the hippocampus were selected, because NMDA receptors are highly expressed in this region. Purified IgG fractions from AAVlac rats showed low level background immunoreactivity in control hippocampal sections (Fig. 8K). In contrast, IgG purified from AAVNMDAR1 rats showed specific signals in CA1, CA3 and the hilus (Fig. 8L), consistent with previous reports on NMDAR1 immunoreactivity in the rat brain and similar to the pattern we observed with a commercial NMDAR1 polyclonal antibody (Fig. 8M).

<u>(iii)</u> <u>Transport of antibodies across the blood-brain barrier and NMDA receptor up regulation.</u>

Transport of the antibodies across the blood-brain barrier and subsequent binding to the native receptor on brain parenchyma tissue was investigated at basal physiological conditions. Groups of AAVlac (n=7) and AAVNMDAR I rats (n=7) were euthanised 4 weeks after peroral dosing of the vaccine and the brains were removed and frozen.

^{3}H-MK-801 autoradiography was used to label the open NMDA receptor channel in hippocampal sections using the technique described by Huettner *et al.* (Huettner *et al.* (1988) *Proc. Natl. Acad. Sci.U. S. A.* 85: 1307-1311) (*see* Example 1O). Three markers of NMDA receptor binding are shown in Fig. 9A-9C. These include ^{3}H-MK-801 autoradiography (Figs. 9A, B), NMDAR1 immunohistochemistry with a commercial antibody (Figs. 9C, D) and *in situ* hybridization with NMDAR1 (Figs. 9E, F), NMDAR2a (Figs. 9G, H) and NMDAR2b (Figs. 9I, J) oligonucleotide probes showed increased binding, immunoreactivity and mRNA levels in AAVNMDAR1-vaccinated (Figs. 9B, D, F, H, J) compared to AAVlac-treated hippocampus (Figs. 9A, C, E, G, I) suggestive of NMDA receptor upregulation. In contrast, hippocampal mRNA levels of the trk B receptor (Figs. 9K, L) in AAVlac (Fig. 9K) and AAVNMDAR1 (Fig. 9L) treated rats were not significantly different. The results showed an increase in ^{3}H-MK-801 binding (arrows) in the hippocampus of AAVNMDAR-vaccinated rats (Figs. 9A,B). Relative density measurements from film autoradiograms showed there was a significant increase in soma and dendritic binding in the CA1 hippocampal region of AAVNMDAR1 compared to AAVlac animals (0.281±0.006 vs 0.2580±0.008; p=0.049, Student's t-test) while there was no significant difference in binding in the dentate granule cell or CA3 layer.

A commercial NMDA receptor-specific antibody was used to determine whether increased ^{3}H-MK-801 binding was associated with an increase in NMDAR 1 protein expression. NMDAR1 rats had a significantly higher level of NMDARl-immunoreactivity in the hippocampus (0.342±0.005 vs 0.314±0.002, p=0.0009, Student's t-test) (Figs. 9C,D). Moreover, *in situ* hybridization using probes to NMDAR1, NMDAR2A and NMDAR2B subunits and density measurements of film autoradiograms also showed a highly significant increase in NMDAR1 (0.462±0.01 vs 0.356±0.016, p=0.0005) as well as the NNDAR2A (0.430±0.023 vs 0.378±0.009, p=0.038) and NNDAR2B (0.480±0.01 vs 0.430±0.015, p=0.019) mRNA (Figs. 9E-J).

The immunization effects were specific to these NMDAR mRNAs as no difference in mRNA levels of the trk B receptor were observed in either the dentate granule cell (AAVlac - 0.310±0.009 vs AAVNMDAR1 0.314±0.005, p=0.706) or CA1 layer (0.308±0.009 vs 0.318±0.006, p=0.394) (Figs. 9K-L). The changes in

MK-801 binding, together with the increased NMDA receptor subunit transcripts and NMDA receptor immunoreactivity indicate passage of the antibody across the blood-brain barrier and partial antagonism of the receptor with a compensatory upregulation of NMDA receptors.

(iv) Modulation of Krox-24 Transcription Factor

To demonstrate that the antibodies are capable of indirectly modulating events involving the NMDA receptor, an indirect marker of NMDA receptor antagonism was examined. The expression of Krox-24 protein, a transcription factor whose cortical expression is maintained under tonic NMDA receptor activation, was investigated.

Fig. 10A-10H are sections of the cortex of animals. Immunchistochemistry analysis shows reduction of basal levels of Krox-24 protein within the cortex in the AAVNMDAR1-treated (Fig. 10C) compared to AAVlac-treated (Fig. 10B) or naïve animals (Fig. 10A). Insets show high-powered images of Krox-24 immunoreactivity. Anti-rat IgG immunohistochemistry used to investigate BBB penetration of IgG under basal conditions showed a weak but increased level of immunoreactivity in the cortex of AAVNMDARl-treated (Fig. 10F) when compared to AAVlac (Fig. 10E) or naïve (Fig. 10D) animals. IgG penetration was significantly enhanced 90 min following kainate treatment, with specific increases in CA2-CA3 hippocampal regions (arrows, Fig. 10E) compared to the same region under basal conditions (arrows, Fig. 10H). Fig. 10I shows an immunoblot analysis of CSF sampled from an AAVlac (Lane 1) and AAVNMDAR1 animal (Lane 2) showing detection of a 117 ka protein species and breakdown products only from AAVNMDAR1 CSF under basal conditions. Increased levels of these proteins were found 90 min following kainate treatment (Lane 3).

The results demonstrate that antagonism of NMDA receptors using MK-801 leads to a significant decrease in Krox-24 expression. In AAVNMDAR1 (n=6) but not AAVlac (n=5) or naïve control rats (n=4), a 21±3.5% (p=0.038, NMDAR1 vs. lac, Student's t-test) a decrease in Krox-24 protein expression was observed in the cortex (Figs. 10A-C).

To further confirm passage of autoantibodies across the blood-brain barrier, the CSF was screened for the presence of NMDAR1 antibodies as described in Example 3(ii). Low levels of a 117kD protein, consistent with the molecular weight of the native

NMDAR 1 receptor subunit and breakdown products similarly recognized by commercial antibodies were detected in the CSF (Fig 10I). Furthermore, levels of these protein species were increased following kainate treatment. In addition, brain sections of immunized rats were stained with an anti-rat IgG antibody to look at immunoglobulin transport into brain parenchyma. Under resting conditions, an increase in brain anti-IgG binding was observed in AAVNMDAR1 rats (Figs. 10F) compared to naïve or AAVlac rats (Figs. 10D,E). Following kainate administration, a further increase in the anti-IgG immunoreactivity was apparent in hippocampal sections from AAVNMDAR1 rats (Figs. 10G-H) consistent with the CSF immunoblot result.

(v) Effect of the vaccine on inflammation in the brain

To determine whether there was any inflammatory response, cellular infiltrate or microglial reaction due to vaccination with AAVNMDAR1 vaccination, sections of the brain were analysed. Fig. 11A-11I demonstrate that no inflammatory responses was observed in the brain. Similar levels of basal isolectin-B4 (Figs. 11A-C), OX-18 (Figs. 11D-F) and CD8 (Figs. 11G-I) immunoreactivity in the cortex of naïve (Figs. 11 A,D,G), AAVlac (Figs. 11B,E,H), AAVNMDAR1 (Figs. C,F,I) suggested no inflammatory responses associated with vaccination. Scale A-1, 500 μm. The results showed that brain morphology appeared normal under light microscopy in vaccinated rats, and immunohistochemistry using antibodies to isolectin B4, a microglial marker (Streit, *et al.* (1987) *J. Neurocytol.* 16: 249-60), OX-18, an MHC Class I marker, and CD8, a cytotoxic T-cell marker, showed no differences between AAVNMDARl, AAVlac and naïve control animals (Figs. 11A-I). The data therefore demonstrates the successful and stable NMDAR1 transgene expression over a period of five months without an inflammatory response.

In summary, the results demonstrate that purified antibodies from AAVNMDAR1 rats specifically bound to hippocampal neurons and the pattern of immunoreactivity mirrored that obtained using commercial NMDA receptor antibodies. Furthermore, antibodies from AAVNMDAR1 rats specifically bound to, and antagonized the NMDA-induced increase in the calcium signal in primary neurons, whereas serum from naïve rats or from rats immunized with the control AAVlac vector did not. Attenuation of the calcium signal suggests that the autoantibodies bind to and inhibit NMDA receptors.

Indirect support for *in vivo* antagonism of NMDA receptors was demonstrated by examining the change in expression of the transcription factor, Krox-24. Notably, the basal expression of Krox-24 has previously been shown to be under glutamatergic tonic activation and pharmacological antagonism of NMDA receptors inhibit cortical levels of the protein (Gass *et al.* (1993) *Neuroscience* 53: 749-758). AAVNMDAR1-immunized animals had a -20% decrease in basal Krox-24 expression consistent some passage of the antibody and inhibition of NMDA receptor mediated glutamatergic neurotransmission.

Additional evidence of blood-brain barrier passage and receptor antagonism was shown by the upregulation of NMDAR1 as well as NMDAR2A and NMDAR2B mRNA in hippocampal sections from AAVNMDAR1-immunized animals. This increase in the NMDA receptor subunit mRNA transcripts was associated with an increase in NMDA receptor protein, a result consistent with previous reports of increased NMDA receptor subunit mRNA levels following pharmacological antagonism of the receptor (Wilson *et al.* (1998) *Dev. Brain Res.* 109: 211-220). Even though the vaccine targeted just the NMDAR1 subunit, the NMDA receptor *in vivo* is not a homomeric complex composed of NNMAR1 subunits, but is invariably a heteromer made up with NMDAR2 subunits (Sheng *et al.* (1994) *Nature* 368: 144-147 and McBain *et al.* (1994) *Physiol. Rev.* 74: 723-760). Moreover, the expression of NMDARl and NMDAR2 subunits to be appear linked, and functional antagonism of the receptor leads to an upregulation of both NMDAR1 and NMDAR2 subunits (Wilson *et al.* (1998) *supra*; Fossom *et al.* (1995) *Mol. Pharmacol.* 48: 981-987). The results also showed no difference in hippocampal mRNA levels of NR2C, NR2D or the trk B receptor between AAVlac and AAVNMDAR1-immunized rats suggesting that the vaccination effects were specific to NMDA receptors.

Collectively, these results demonstrate that the genetic vaccine of the invention can be used for functional genomic studies to investigate the function of proteins expressed in the brain. The results describe a simple genetic vaccine to induce autoantibodies which target the gene of interest and thereby modify its function.

Example 7: Vaccination effects on rat learning and memory (Cognition)

To investigate the effect of the AAVNMDAR1 vaccine on learning and memory, the ingestive behaviour and body weights of rats vaccinated with the genetic vaccine were monitored. At weekly intervals following vaccination with no alteration in feeding or weight gain in either group.

At four months post-vaccination, rats underwent behavioural testing as described in Example 1R. One of the most sensitive behavioural tests for NMDA antagonists is impairment of performance in tasks that involve hippocampal-dependent learning and memory. NMDA receptor antagonists at effective neuroprotective doses typically interfere with learning in spatial navigation tasks (Bannerman, *et al.* (1995) *Nature* 378: 182-186), although newer generation compounds, particularly NMDA receptor antagonists that are partial agonists at the glycine binding site may facilitate learning in hippocampal tasks.

AAVNMDARl-vaccinated rats were compared with AAVlac rats to determine whether the immunization was associated with impairment in a spatial maze task (Barnes (1979) *J. Comp. Physiol. Psychol.* 93: 74-104). Fig. 12. shows the results of AAVNMDAR1 vaccination effects on learning and memory.

Fig. 12A is a graph showing errors and latencies recorded on the Barnes Circular Maze from AAVlac-treated rats, and AAVNMDAR1-vaccinated rats. Fig. 12B is a graph showing the results from the line crossing and circular track mobility tests. Data represents the number of line crossings in 5 min intervals over 5 successive days in AAVlac-treated, or AAVNMDAR1-vaccinated rats. In the circular track test, the number of completed circuits in successive days for AAVlac-treated and AAVNMDAR1-vaccinated animals are represented. Fig. 12C depicts the results from the contextual fear conditioning for AAVlac-treated and AAVNMDAR1-vaccinated animals (*p=0.025). Fig. 12D depicts the results from the Spontaneous Object Recognition test. The left graph is a comparison within groups of time spent exploring during the sample phase (Al vs A2) and the choice phase (A3 vs B). The right graph is a comparison between groups of total time spent exploring in sample phase (A1+A2), choice phase (A3+B), and the discrimination index (B-A3). (* p=0.041).

Results showed that in the Barnes maze the AAVNMDARl rats (n=15) had significantly improved performance compared to AAVlac rats (n=16) as defined by reduced latencies to enter the escape box (repeated measures ANOVA, p=0.043, Fig. 12A). Improved performance in the Barnes maze may be due to other factors such as increased mobility. To examine increased mobility, the rats were tested on circular track and line crossing mobility paradigms, both of which failed to demonstrate a difference between the groups (repeated measures ANOVA, p=0.87 and p=0.32 respectively, Fig. 12B).

NMDA receptor activation has also been demonstrated to be involved in the storage of other forms of memory, such as contextual memory (Kiyama *et al,* (1998) *J. Neurosci.* 18; 6704-6712 and object recognition memory (Puma *et al.* (1998) *Neurosci. Lett.* 244: 97-100). To assess whether the vaccinated rats had an improved contextual memory, the rats were tested for their freezing responses. The results demonstrated that AAVNMDAR1-vaccinated rats (n=12) exhibited a stronger freezing response than AAVlac rats (n=10) when placed in an environment in which a mild electric shock had been previously received (p=0.025, Fig. 12C). In addition, AAVNMDAR1 rats (n=19) discriminated and explored a novel object for significantly longer than an object previously encountered compared to AAVlac rats (n=16), (p=0.041, Fig. 12D).

These surprising results demonstrate the significant improvement in learning and memory in the AAVNMDAR1-vaccinated rats. The result obtained using the hippocampally-dependent Barnes circular platform task (Barnes, 1979, *supra*) was also generalizable to a novel object recognition task (Puma *et al.*, 1998, *supra*), and a contextual association task ((Kiyama *et al.* (1998) *supra*). The improved learning and memory observed in the present invention may be due to the increase in the NMDA receptor number, perhaps compensating for antagonism of the receptor. Increases in NMDA receptor expression mediate experience-dependent synaptic plasticity in the visual cortex (Quinlan *et al.* (1999) *Nat Neurosci.* 2: 352-357). Furthermore, transgenic mice which overexpress NMDAR2B and NMDAR1 in the forebrain exhibit improved learning and memory associated with facilitation of synaptic potentiation (Tang *et al.* (1999) *Nature* 401: 63-69).

The gain of function demonstrated by the significant improvement in learning and memory, may be associated with an increase in transcription and translation of the targeted gene. Targeted autoimmunity using genetic vaccines may therefore provide not only the novel therapies to treat neurological disorders, but also a tool to analyze the function of genes.

Example 8: Vaccination effect on nociception

To investigate the effect of vaccination on pain tolerance associated with NMDA receptors, the tail-immersion and hot-plate pain assays were employed (Taulbee and Kasting (1988) *J. Pharmacol. Methods* 20: 197-206) (*see* Example 1S). These assays were used to determine whether AAVNNDAR1 immunized rats had altered responses to painful stimuli. The NMDA receptors have also been implicated in nociception (Nasstrom *et al.* (1992) *Eur. J. Pharmacol.* 212, 21-29). NMDA receptors mediate the release of substance P in the spinal cord (Liu *et al.* (1997) *Nature* 386: 721-724) and direct intrathecal administration of NMDA lowers pain thresholds (Davis *et al.* (1999) *J. Pharmacol. Exp. Ther.* 289: 1048-1053). Conversely, NMDA receptor antagonists show analgesic activity (Coderre (1993) *Eur. J. Neurosci.* 5: 390-393).

Fig. 13 depicts the results vaccination effects on Nociception. The latency for escape responses for the tail immersion test and latency for escape responses or hindpaw licking in the hot plate test for AAVlac (black bars) and AAVNMDAR1 (white bars) animals. Each bar represents the mean±SEM for all animals in that group (* $p=0.04$ for tail immersion and $p=0.02$ for hot plate tests, Student's t-test).

The results demonstrated that following tail immersion, AAVNMDAR1 rats ($n=10$) showed significantly longer latency to tail-flick than the AAVlac control rats ($n=8$), ($p=0.04$, Fig. 13). Similarly, in the hot plate assay, the latency of the AAVNMDAR1 rats ($n=8$) was greater than the AAVlac animals ($n=8$), ($p=0.02$, Fig. 13).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A therapeutic agent comprising (A) a therapeutically effective amount of an antigen capable of eliciting the production of antibodies in the circulatory system of the subject, the antigen being chosen such that the antibodies, produced in response thereto, bind to, and modify the function of a target protein in the central nervous system or (B) a therapeutically effective amount of an isolated antibody, or an antibody portion, wherein the antibody, or an antibody portion binds to, and modifies the function of a target protein in the central nervous system.

2. The agent of claim 1, wherein the antigen further comprises an antigen chosen such that the antibodies, produced in response thereto, pass across the blood-brain barrier into the central nervous system.

3. The agent of claim 1, wherein the antigen further comprises an antigen chosen such that the antibodies, produced in response thereto, (A) ameliorate or prevent at least one neurological disorder selected from the group consisting of epilepsy, stroke, Alzheimer's disease, Parkinson's disease, dementia, Huntington's disease, amyloid lateral sclerosis and depression, (B) to ameliorate the neuroendocrine disorder, or (C) improve cognition of a subject.

4. The agent of claim 1, wherein the antigen further comprises an antigen chosen such that the antibodies, produced in response thereto, ameliorate or prevent stroke.

5. The agent of claim 1, wherein the antigen further comprises an antigen chosen such that the antibodies, produced in response thereto, ameliorate or prevent epilepsy.

6. The agent of claim 1, wherein the antigen further comprises an antigen selected from the group of neurotransmitters, neuroreceptors, transporters, ion channels, signal transduction molecules, enzymes involved in the synthesis or degradation of neurotransmitters, growth factors, transcription factors, and cell surface molecules.

7. The method of claim 6, wherein the antigen is an NMDA receptor and, optionally, the antibodies, produced in response thereto, decrease Krox-24 expression.

8. The method of claim 7, wherein the antigen is NMDAR1.

9. The agent of claim 1, wherein the agent further comprises a vaccine selected from the group consisting of viral vector vaccines, DNA vaccines, a peptide vaccines, crude antigen vaccines, or a combination thereof.

10. The agent of claim 9, wherein the vaccine is a viral vector vaccine comprising a viral vector selected from the group consisting of an RNA viral vector and a DNA viral vector.

11. The agent of claim 10, wherein the viral vector vaccine comprises a viral vector selected from the group consisting of an adenovirus vector, a herpes virus vector, a parvovirus vector, and a lentivirus vector.

12. The agent of claim 11, wherein the viral vector is an adeno-associated virus vector.

13. The agent of claim 1, wherein the therapeutically effective amount of an isolated antibody, or an antibody portion, further comprises an antibody, or an antibody portion elicited in a mammal for administration to the subject.

14. The agent of claim 1, wherein the isolated antibody, or antibody portion is administered is chosen such that it can pass across the blood-brain barrier into the central nervous system.

15. The agent of claim 1, wherein the isolated antibody, or an antibody portion is selected from the group consisting of a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a chimeric antibody, a humanized antibody, an Fab fragment, an $F(ab')_2$ fragment and a single chain Fv fragment.

16. The agent of claim 1, wherein the isolated antibody, or antibody portion is selected from the group consisting of an anti-NMDA antibody, an anti-GluR antibody, an anti-NK-1 antibody, an anti-dopamine transporter antibody and anti-glutamic acid decarboxylase antibody.

17. The agent of claim 1, wherein the isolated antibody, or an antibody portion is an anti-NMDA antibody and, optionally, the isolated antibody decreases Krox-24 expression.

18. The agent of claim 17, wherein the isolated antibody, or an antibody portion is an anti-NMDAR1 antibody.

19. The agent of claim 1, wherein the isolated antibody, or antibody portion is an anti-GluR antibody.

20. The agent of claim 19, wherein the isolated antibody is an anti-GluR4 antibody.

20. The agent of claim 19, wherein the isolated antibody is an anti-GluR6 antibody.

21. The agent of claim 3, wherein the agent further comprises an antigen chosen such that the antibodies, produced in response thereto, ameliorate obesity.

22. The agent of claim 21, wherein the antigen is selected from the group consisting of neuropeptide-Y (NPY), galanin, cocaine-and amphetamine-regulated transcript (CART), orexin, thyrotropin - releasing hormone (TRH), leptan, corticotropin - releasing hormone (CRH) and pro-opiomelanocortin (POMC).

23. The agent of claim 22, wherein the antigen is neuropeptide Y.

24. The agent of claim 22, wherein the antigen is galanin.

25. The agent of claim 1, wherein the isolated antibody, or antibody portion is selected from the group consisting of anti-NPY antibodies, anti-galanin antibodies, anti-CART antibodies, anti-orexin antibodies, anti-TRH antibodies, anti-leptan antibodies, anti-CRH antibodies, and anti-POMC antibodies.

26. The agent of claim 25, wherein the antibody is an anti-NPY antibody.

27. The agent of claim 25, wherein the antibody is an anti-galanin antibody.

28. The agent of claim 1, wherein the target protein is selected from the group of neurotransmitters, neuroreceptors, transporters, ion channels, signal transduction molecules, enzymes involved in the synthesis or degradation of neurotransmitters, growth factors, transcription factors and cell surface molecules.

29. The agent of claim 28, wherein the target protein is selected from the group consisting of NPY neuropeptide and galanin.

30. The agent of claim 1 wherein the agent further comprises a pharmaceutical acceptable carrier.

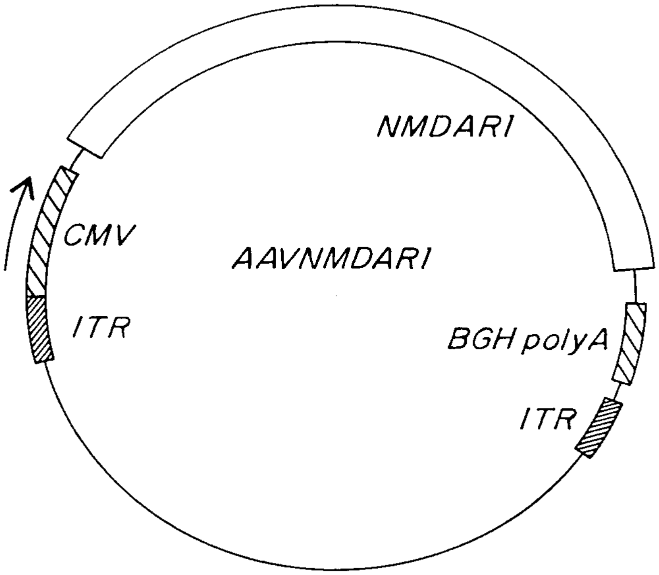


FIG. 1A

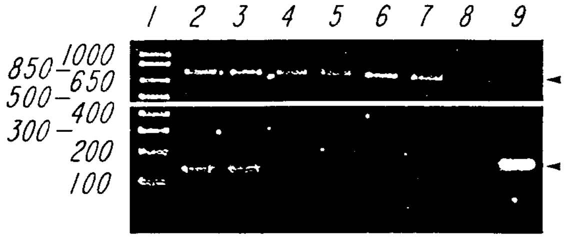


FIG. 1B

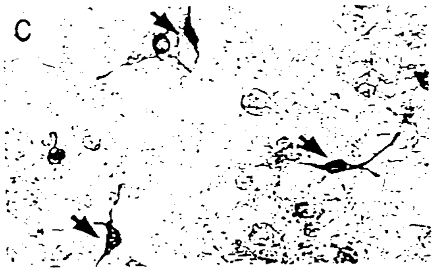


FIG. 1C

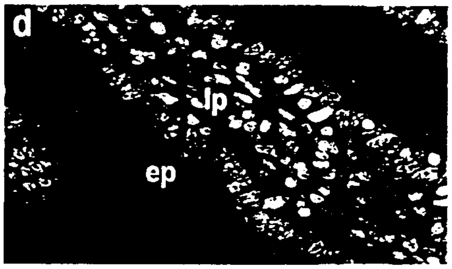


FIG. 1D

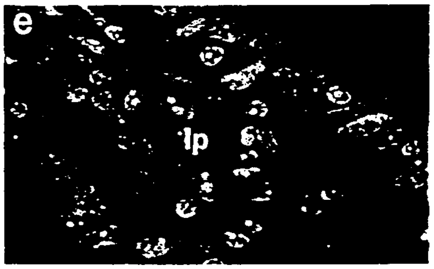


FIG. 1E

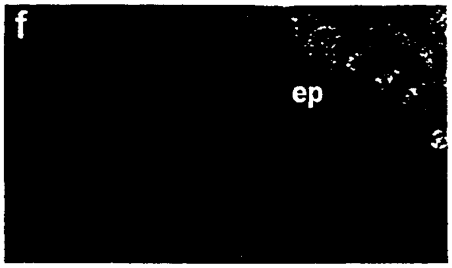


FIG. 1F

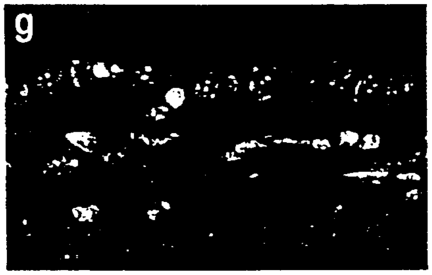


FIG. 1G

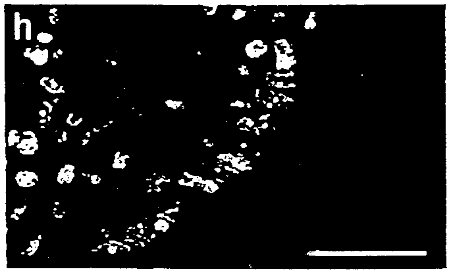


FIG. 1H

FIG. 2A

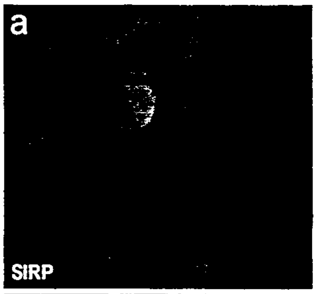


FIG. 2B

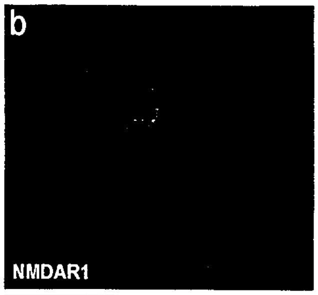


FIG. 2C

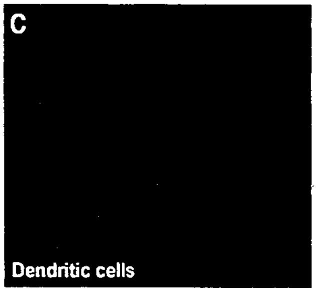


FIG. 2D

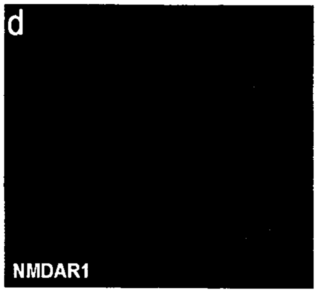


FIG. 2E

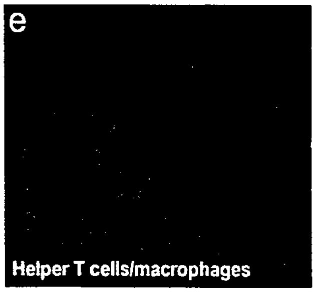


FIG. 2F

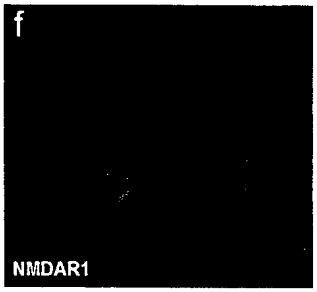


FIG. 2G

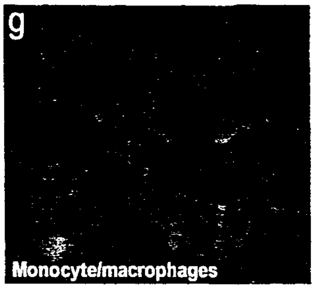
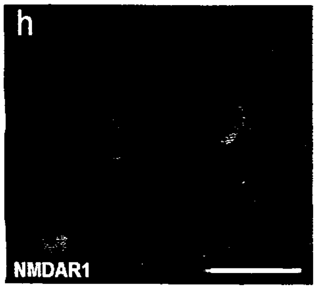


FIG. 2H



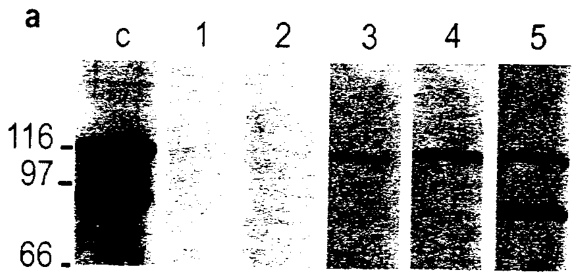


FIG. 3A

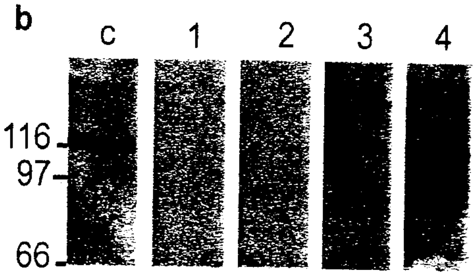


FIG. 3B

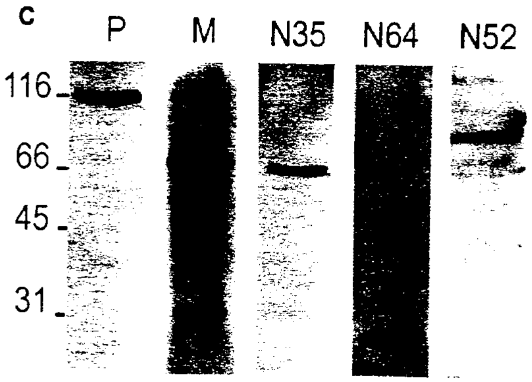


FIG. 3C

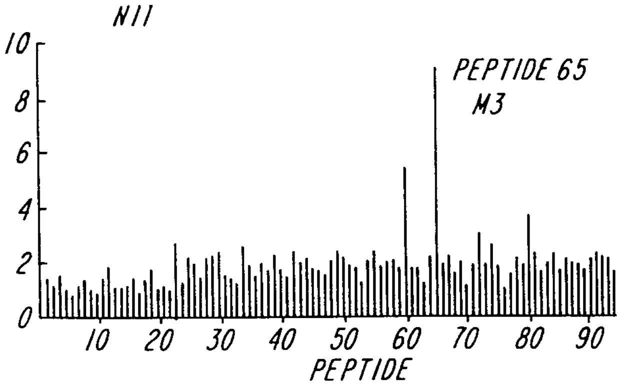


FIG. 3D

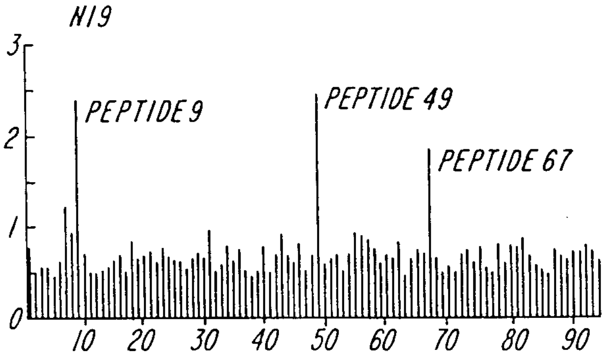


FIG. 3E

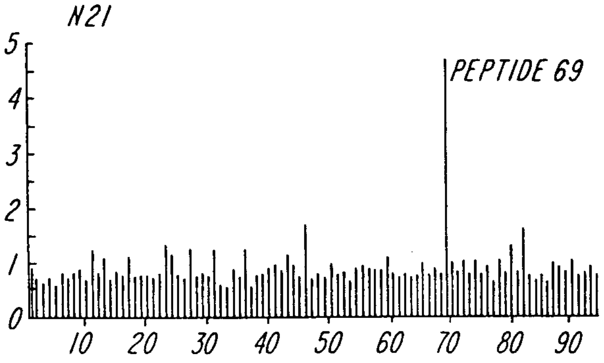


FIG. 3F

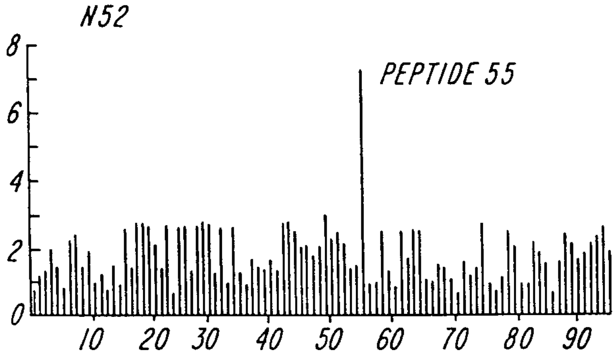


FIG. 3G

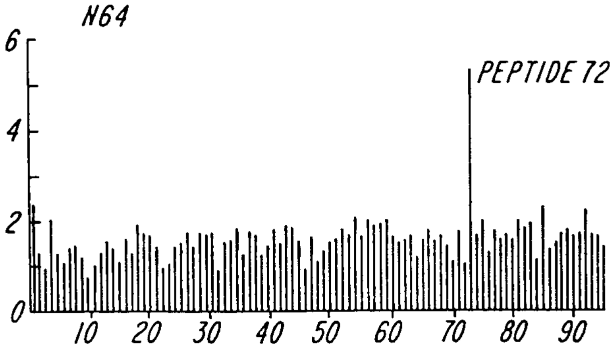


FIG. 3H

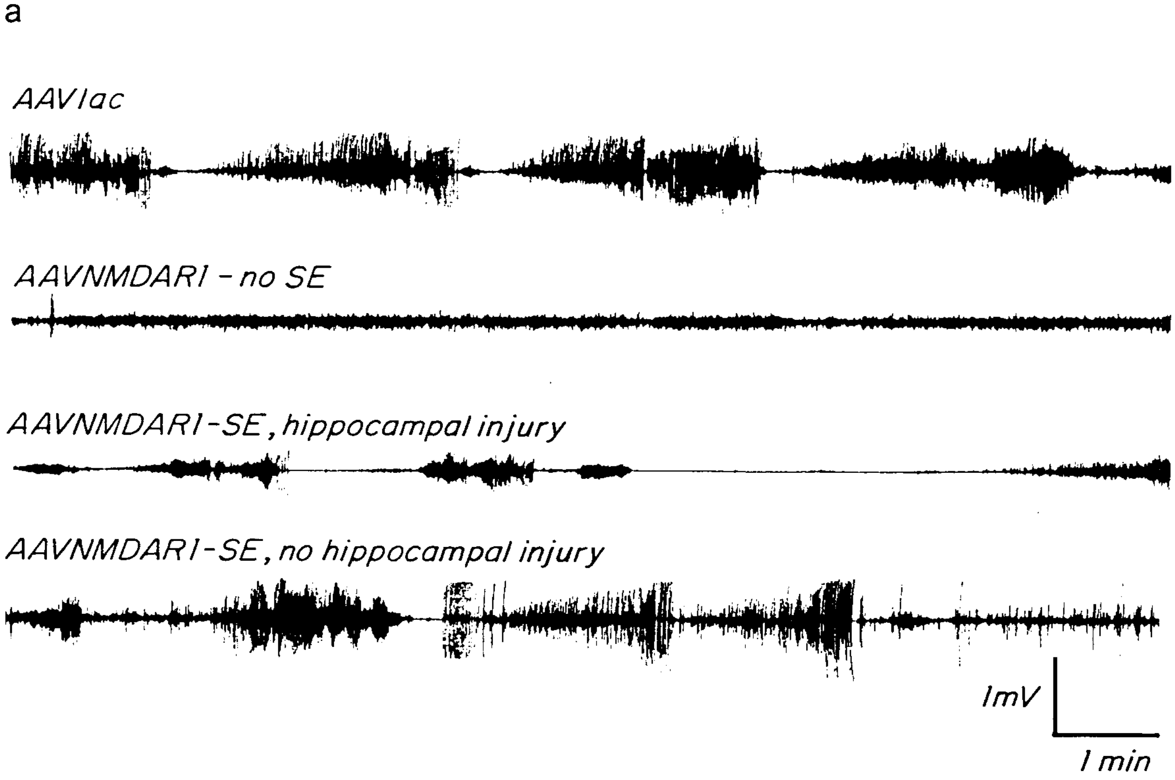

a
AAVlac
AAVNMDAR1 - no SE
AAVNMDAR1-SE, hippocampal injury
AAVNMDAR1-SE, no hippocampal injury
1mV
1 min

FIG. 4A

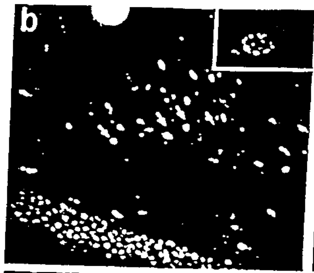


FIG. 4B

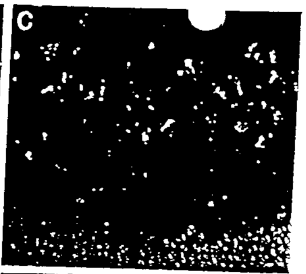


FIG. 4C

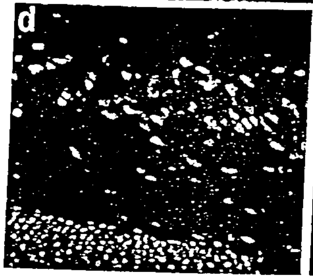


FIG. 4D

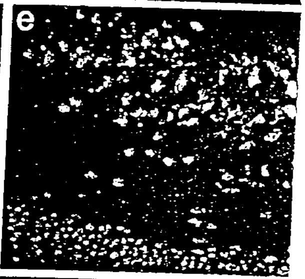


FIG. 4E

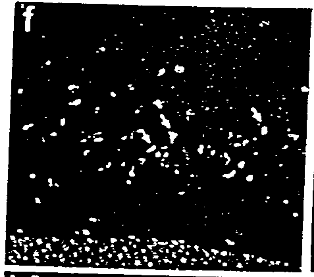


FIG. 4F

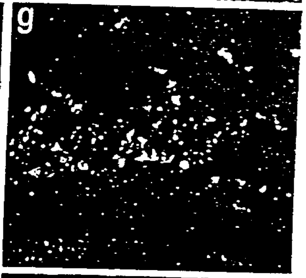


FIG. 4G

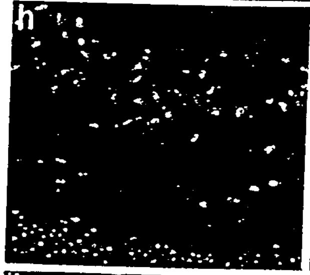


FIG. 4H

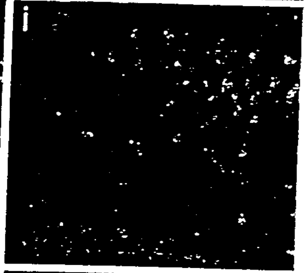


FIG. 4I

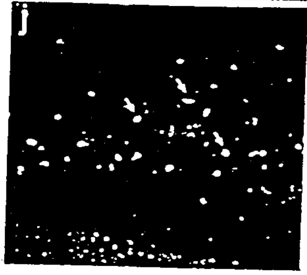


FIG. 4J

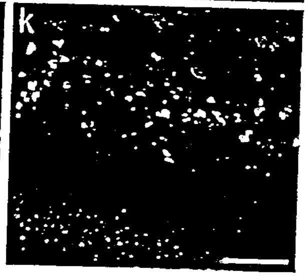


FIG. 4K

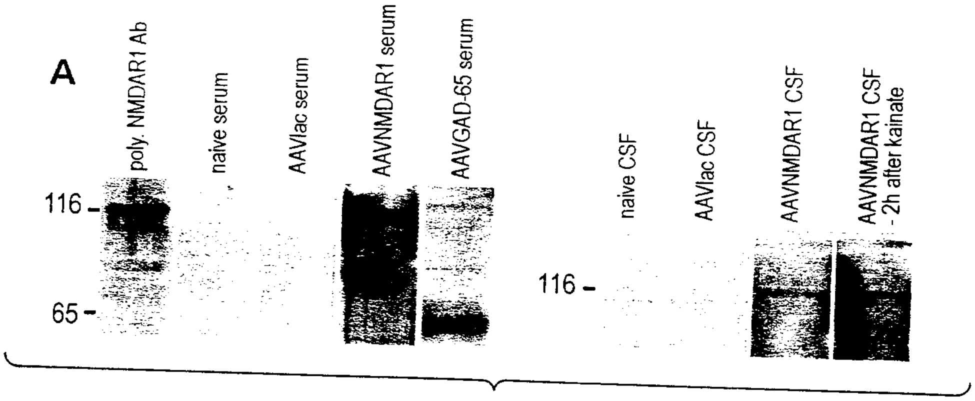


FIG. 5A

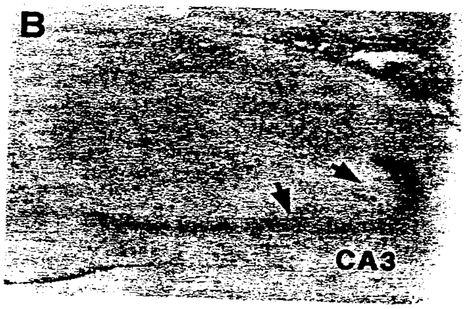


FIG. 5B

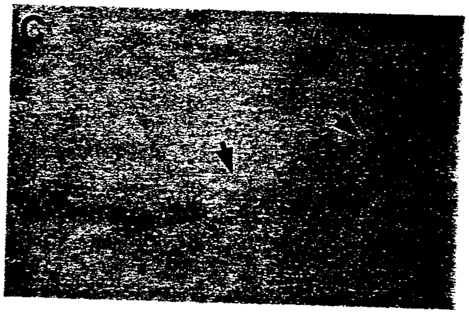


FIG. 5C

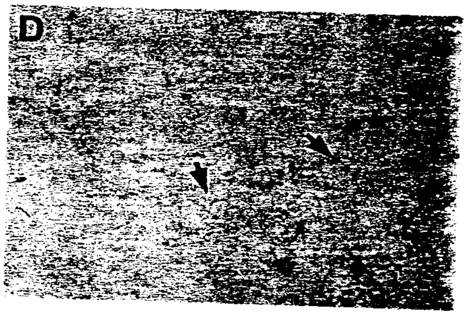


FIG. 5D

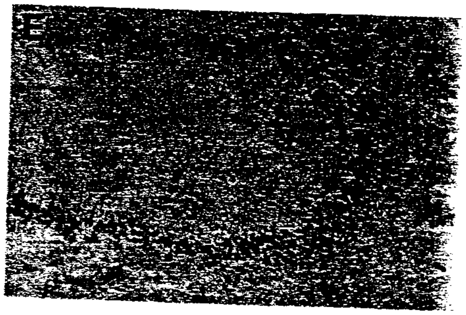


FIG. 5E

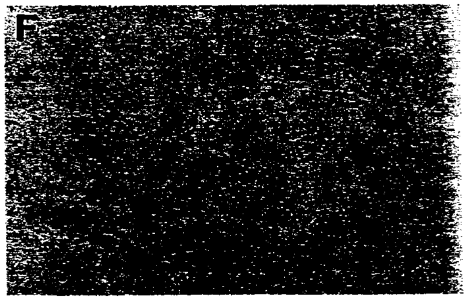


FIG. 5F

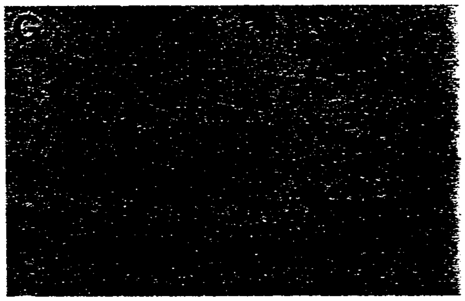


FIG. 5G

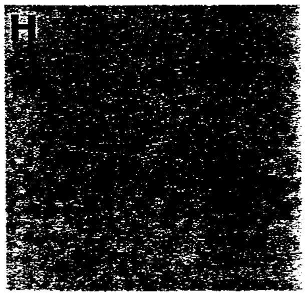


FIG. 5H

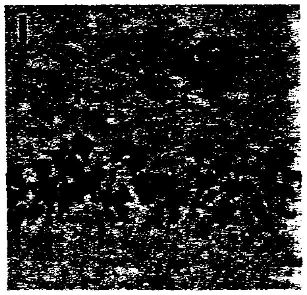


FIG. 5I

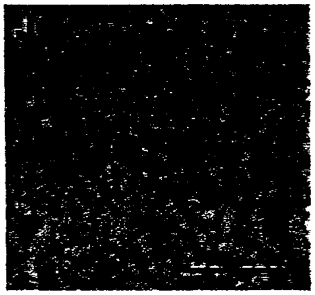


FIG. 5J

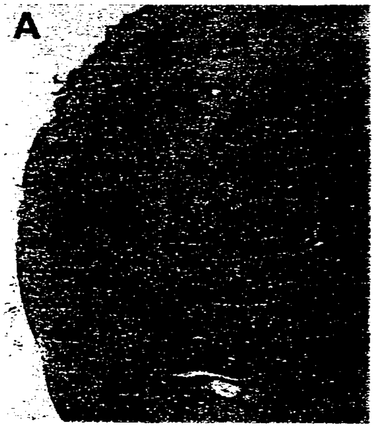


FIG. 6A

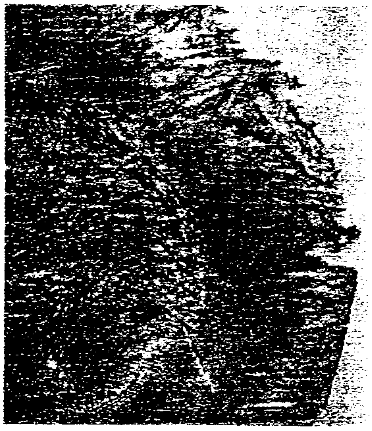


FIG. 6B

FIG. 6C

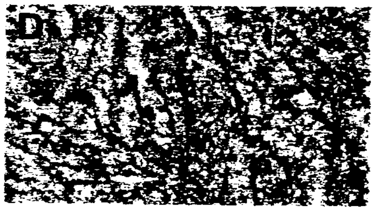


FIG. 6D

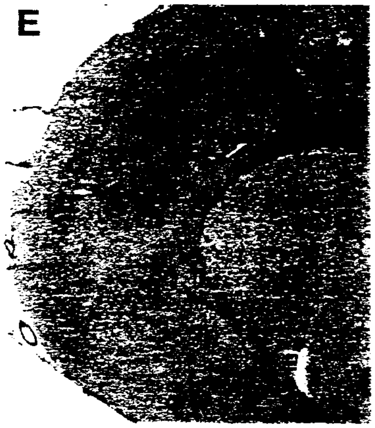


FIG. 6E

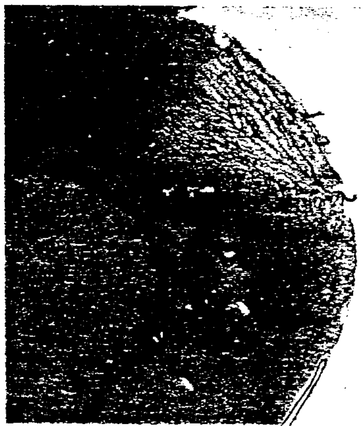


FIG. 6F

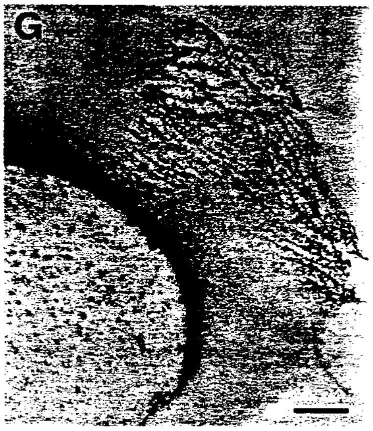


FIG. 6G

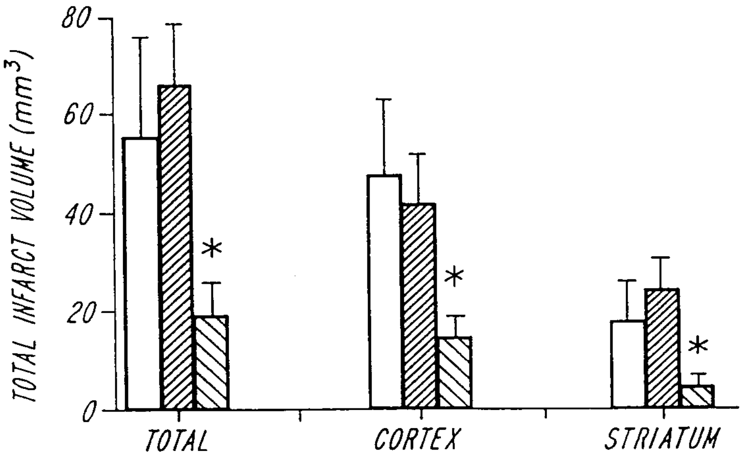


FIG. 6H

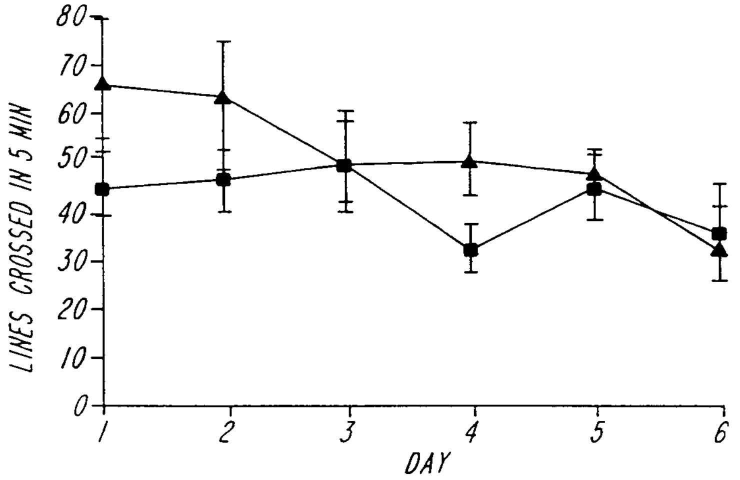

LINES CROSSED IN 5 MIN
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
DAY

FIG. 7A

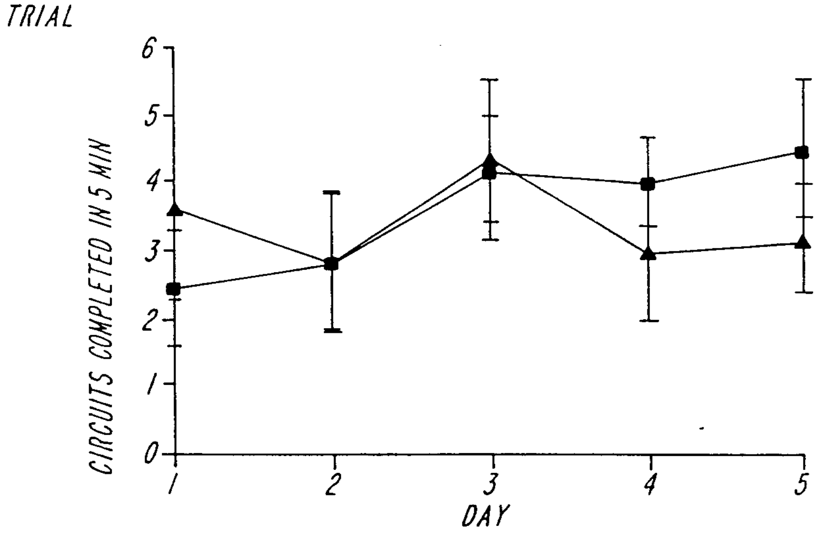

TRIAL
CIRCUITS COMPLETED IN 5 MIN
6
5
4
3
2
1
0
1
2
3
4
5
DAY

FIG. 7B

FIG. 8A

FIG. 8B

FIG. 8C

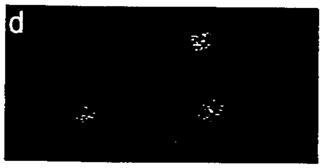


FIG. 8D

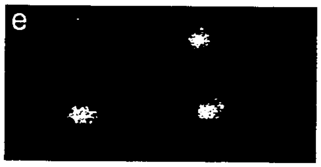


FIG. 8E

FIG. 8F

FIG. 8G

FIG. 8H

FIG. 8I

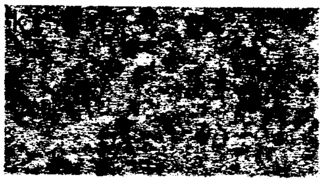


FIG. 8K

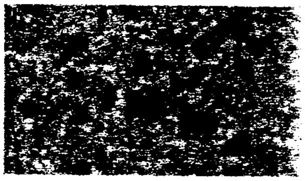
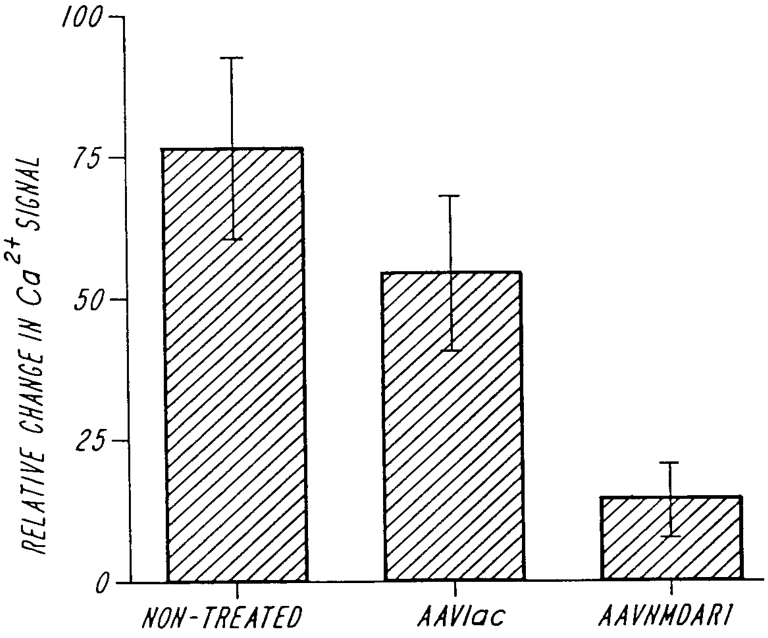


FIG. 8L

FIG. 8M



RELATIVE CHANGE IN Ca^{2+} SIGNAL
100
75
50
25
0
NON-TREATED
AAVlac
AAVNMDAR1

FIG. 8J

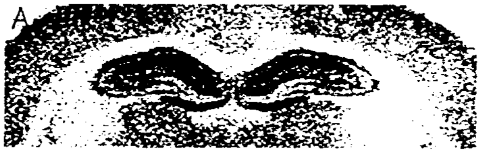


FIG. 9A

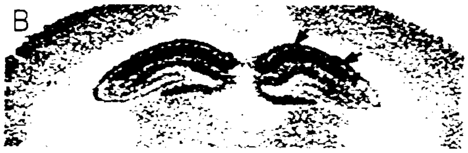


FIG. 9B

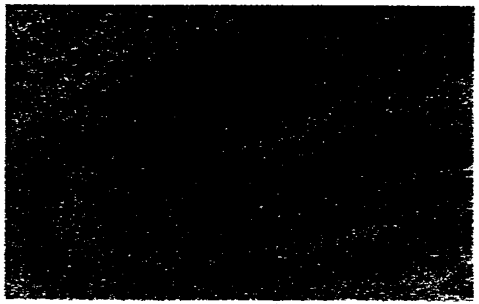


FIG. 9C

FIG. 9D

FIG. 9E

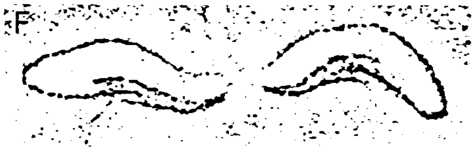


FIG. 9F

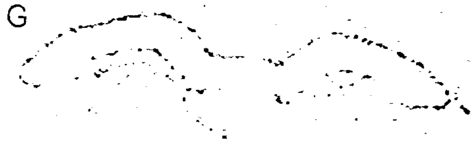


FIG. 9G

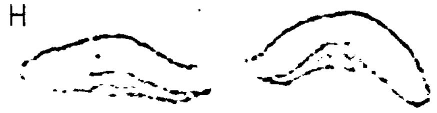


FIG. 9H

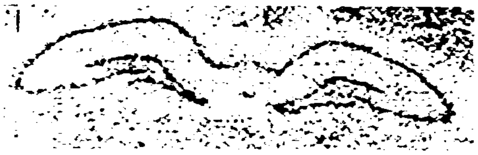


FIG. 9I

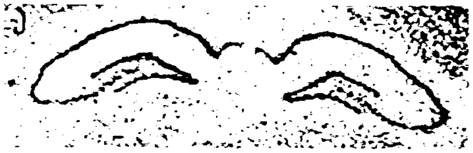


FIG. 9J

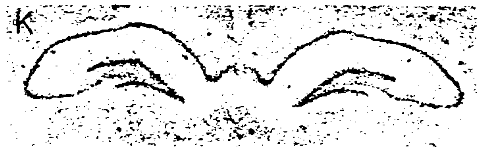


FIG. 9K

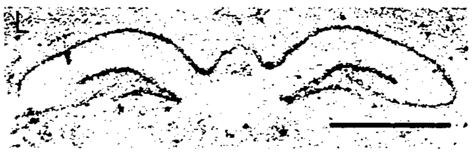


FIG. 9L

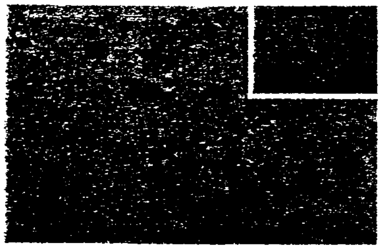


FIG. 10A

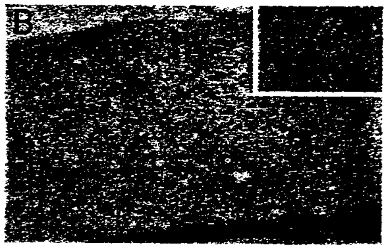


FIG. 10B

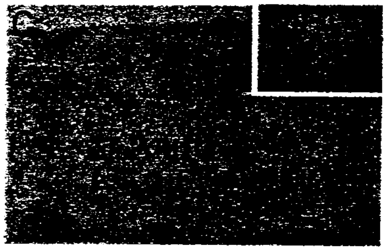


FIG. 10C

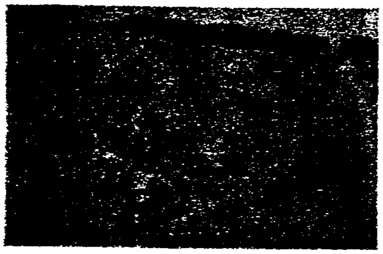


FIG. 10D

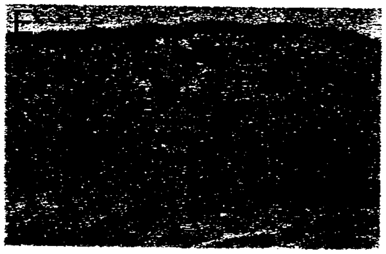


FIG. 10E

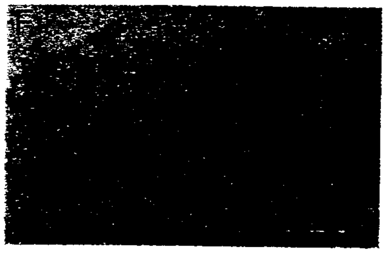


FIG. 10F

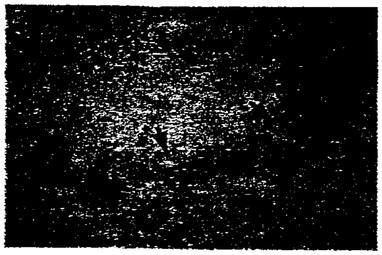


FIG. 10G

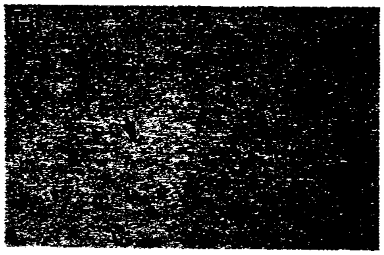


FIG. 10H

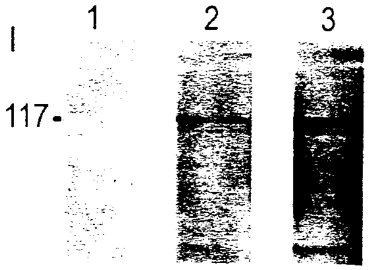


FIG. 10I

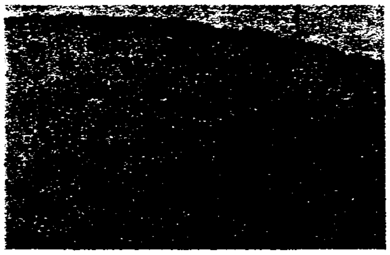


FIG. 11A

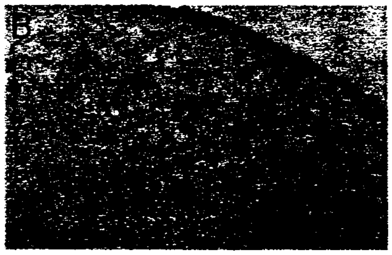


FIG. 11B

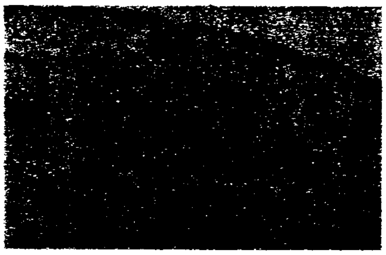


FIG. 11C

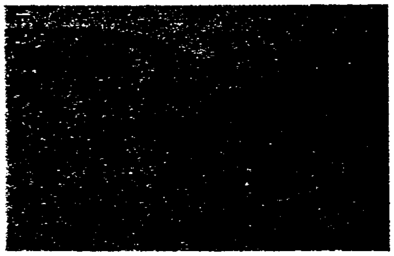


FIG. 11D

FIG. 11E

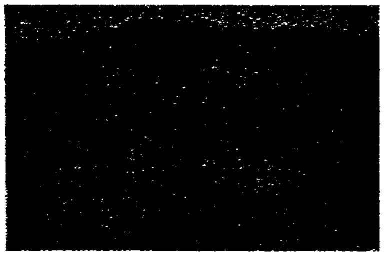


FIG. 11F

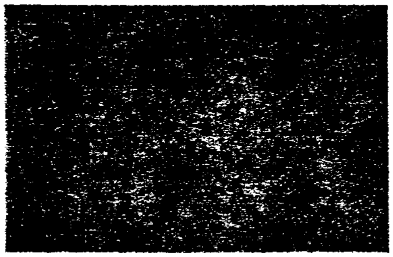


FIG. 11G

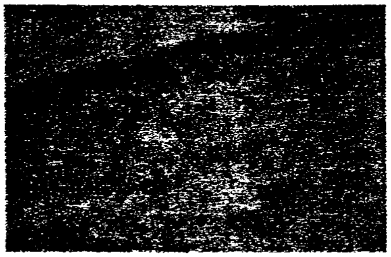


FIG. 11H

FIG. 11I

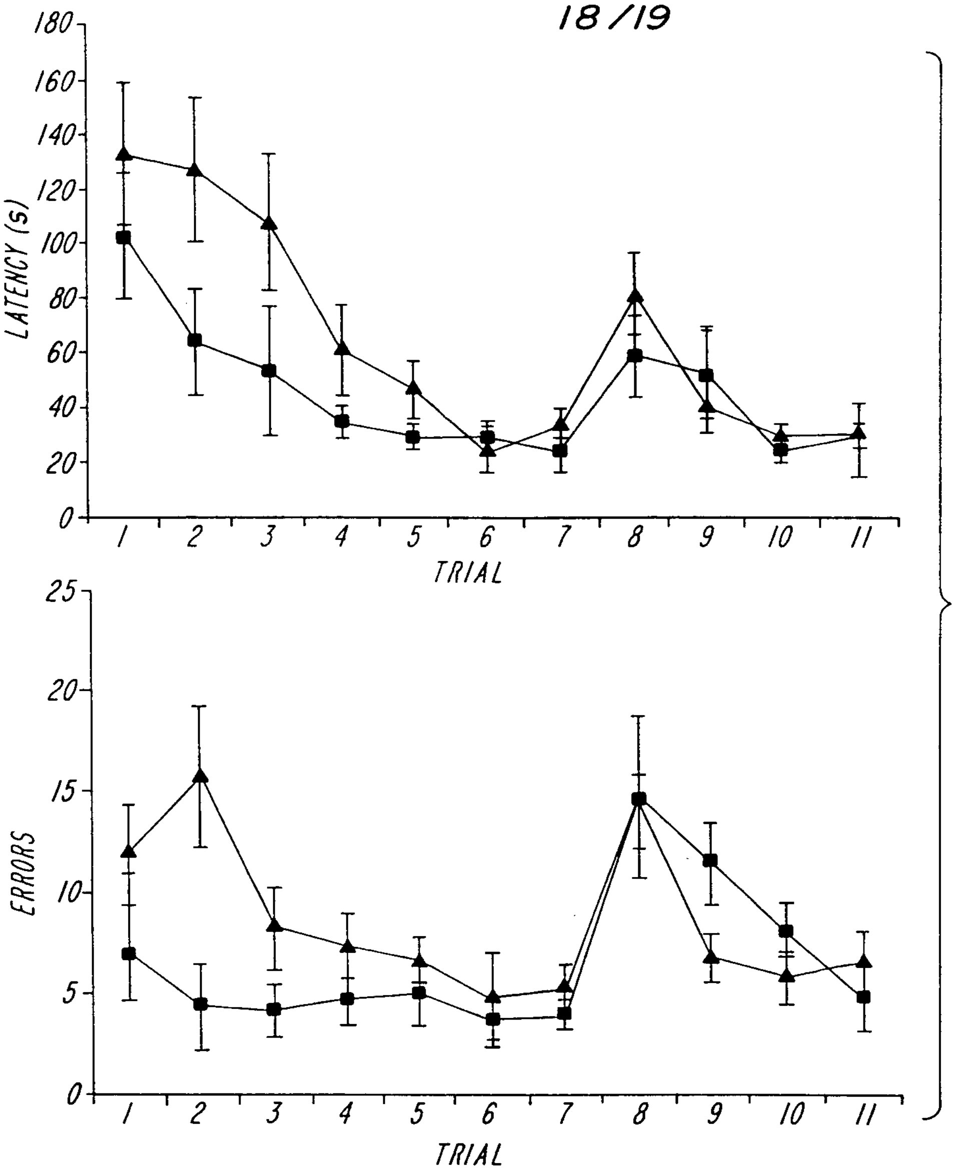


FIG. 12A

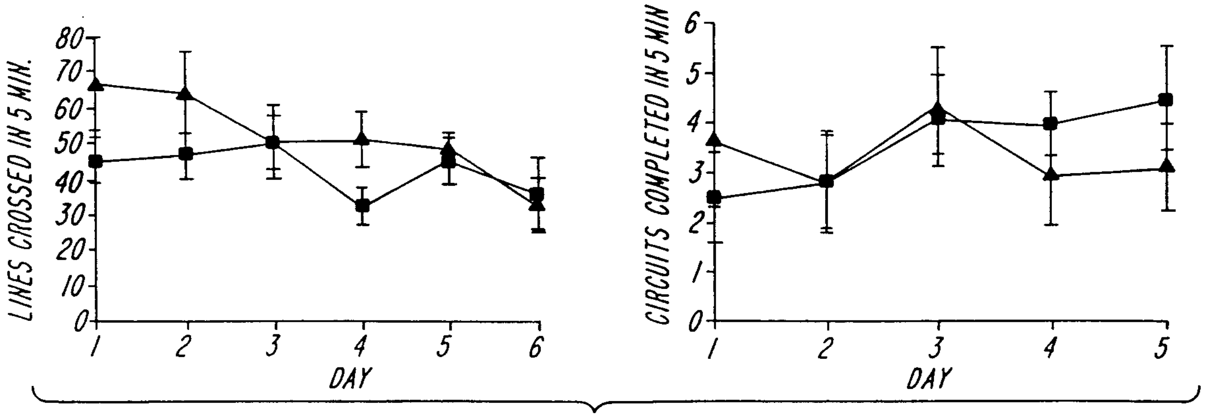


FIG. 12B

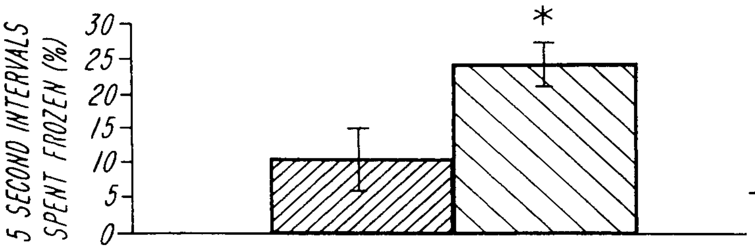


FIG. 12C

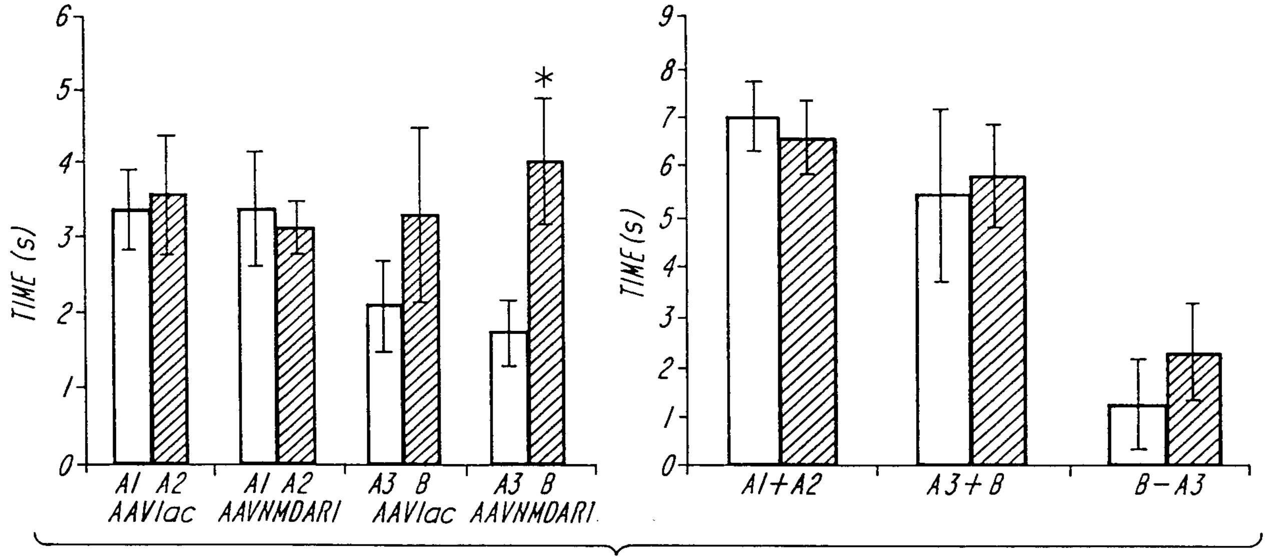


FIG. 12D

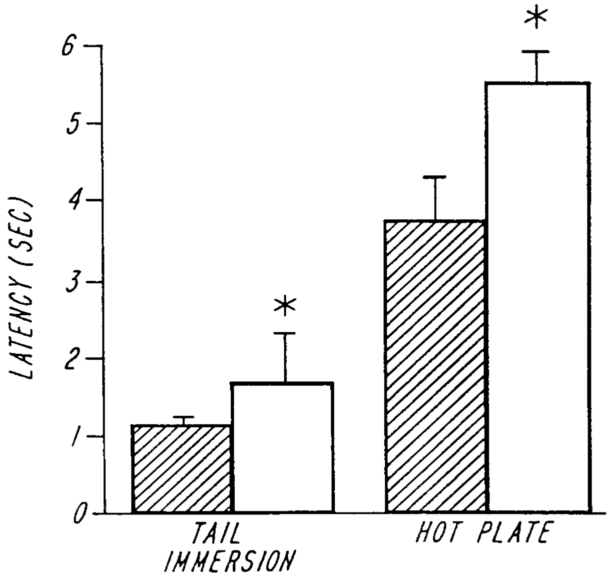


FIG. 13

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

In. ıtional Application No
PCT/US 00/02016

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/00 A61K39/395 A61K48/00 A61P25/00 A61P25/08
A61P25/16 A61P25/24 A61P25/28 //C07K16/28,C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STANTON P K ET AL: "A MONOCLONAL ANTIBODY WHICH MIMICKS GLYCINE ACTION ON N-METHYL-D-ASPARTATE RECEPTORS HAS COMPLEX EFFECTS ON CHANNEL ACTIVATION AND NEURONAL SENSITIVITY TO HYPOXIA" SOCIETY FOR NEUROSCIENCE ABSTRACTS,US,SOCIETY FOR NEUROSCIENCE, vol. 16, no. 1, 1 January 1990 (1990-01-01), page 88 XP002068024 ISSN: 0190-5295 the whole document --- -/--	1,13-18, 30

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search 25 May 2000	Date of mailing of the international search report 19/06/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Covone, M

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INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 00/02016

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	LOBELL ANNA ET AL: "Vaccination with DNA encoding an immunodominant myelin basis protein peptide targeted to Fc of immunoglobulin G suppresses experimental autoimmune encephalomyelitis." JOURNAL OF EXPERIMENTAL MEDICINE MAY 4, 1998, vol. 187, no. 9, 4 May 1998 (1998-05-04), pages 1543-1548, XP002138783 ISSN: 0022-1007 abstract page 1545, right-hand column, line 4-19 table 1 ---	1-3,6,9, 14,30
X	CHARLES, VINOD ET AL: "Atrophy of cholinergic basal forebrain neurons following excitotoxic cortical lesions is reversed by intravenous administration of an NGF conjugate" BRAIN RES. (1996), 728(2), 193-203 , XP000907426 page 200, left-hand column, line 41-51 page 201, left-hand column, line 1-32 ---	1,3,6, 13-15
A	CA 2 087 738 A (PREDDIE RICK E ;BERGMANN JOHANNA E (DE)) 26 July 1994 (1994-07-26) the whole document ---	1-30
A	DURING MATTHEW J ET AL: "Peroral gene therapy of lactose intolerance using an adeno-associated virus vector." NATURE MEDICINE OCT., 1998, vol. 4, no. 10, October 1998 (1998-10), pages 1131-1135, XP002138784 ISSN: 1078-8956 cited in the application the whole document ---	1-30
T	WOOD M.J.A. ET AL: "Neuroprotective autoimmunity a double-edged sword?." NATURE MEDICINE, (2000) 6/4 (383-385). , XP002138785 the whole document ---	1-30
T	DURING MATTHEW J ET AL: "An oral vaccine against NMDAR1 with efficacy in experimental stroke and epilepsy." SCIENCE (WASHINGTON D C). FEB. 25, 2000, vol. 287, no. 5457, 25 February 2000 (2000-02-25), pages 1453-1460, XP000907428 ISSN: 0036-8075 the whole document ---	1-30

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In ational Application No
PCT/US 00/02016

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	"'Oral vaccination against epilepsy and stroke!. SCHLUCKIMPFUNG GEGEN EPILEPSIE UND SCHLAGANFALL." PHARMAZEUTISCHE ZEITUNG, (2 MAR 2000) 145/9 (56-58). , XP000907427 the whole document -----	1-30

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/02016

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA 2087738 A	26-07-1994	NONE	

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(74) Agents: KARA, Catherine, J. et al.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: MODULATORS OF BETA-AMYLOID PEPTIDE AGGREGATION COMPRISING D-AMINO ACIDS

(57) Abstract: Compounds that modulate natural β amyloid peptide aggregation are provided. The modulators of the invention comprise a peptide, preferably based on a β amyloid peptide, that is comprised entirely of D-amino acids. Preferably, the peptide comprises 3-5 D-amino acid residues and includes at least two D-amino acid residues independently selected from the group consisting of D-leucine, D-phenylalanine and D-valine. In a particularly preferred embodiment, the peptide is a retro-inverso isomer of a β amyloid peptide, preferably a retro-inverso isomer of $A\beta_{17-21}$. In certain embodiments, the peptide is modified at the amino-terminus, the carboxy-terminus, or both. Preferred amino-terminal modifying groups alkyl groups. Preferred carboxy-terminal modifying groups include an amide group, an acetate group, an alkyl amide group, an aryl amide group or a hydroxy group. Pharmaceutical compositions comprising the compounds of the invention, and diagnostic and treatment methods for amyloidogenic diseases using the compounds of the invention, are also disclosed.

MODULATORS OF BETA-AMYLOID PEPTIDE AGGREGATION COMPRISING D-AMINO ACIDS

Background of the Invention

Alzheimer's disease (AD), first described by the Bavarian psychiatrist Alois Alzheimer in 1907, is a progressive neurological disorder that begins with short term memory loss and proceeds to disorientation, impairment of judgment and reasoning and, ultimately, dementia. The course of the disease usually leads to death in a severely debilitated, immobile state between four and 12 years after onset. AD has been estimated to afflict 5 to 11 percent of the population over age 65 and as much as 47 percent of the population over age 85. The societal cost for managing AD is upwards of 80 billion dollars annually, primarily due to the extensive custodial care required for AD patients. Moreover, as adults born during the population boom of the 1940's and 1950's approach the age when AD becomes more prevalent, the control and treatment of AD will become an even more significant health care problem. Currently, there is no treatment that significantly retards the progression of the disease. For reviews on AD, see Selkoe, D.J. *Sci. Amer.* , November 1991, pp. 68-78; and Yankner, B.A. *et al.* (1991) *N. Eng. J. Med.* 325:1849-1857.

It has recently been reported (Games *et al.* (1995) *Nature* 373:523-527) that an Alzheimer-type neuropathology has been created in transgenic mice. The transgenic mice express high levels of human mutant amyloid precursor protein and progressively develop many of the pathological conditions associated with AD.

Pathologically, AD is characterized by the presence of distinctive lesions in the victim's brain. These brain lesions include abnormal intracellular filaments called neurofibrillary tangles (NTFs) and extracellular deposits of amyloidogenic proteins in senile, or amyloid, plaques. Amyloid deposits are also present in the walls of cerebral blood vessels of AD patients. The major protein constituent of amyloid plaques has been identified as a 4 kilodalton peptide called β-amyloid peptide (β-AP)(Glenner, G.G. and Wong, C.W. (1984) *Biochem. Biophys. Res. Commun.* 120:885-890; Masters, C. *et al.* (1985) *Proc. Natl. Acad. Sci. USA* 82:4245-4249). Diffuse deposits of β-AP are frequently observed in normal adult brains, whereas AD brain tissue is characterized by more compacted, dense-core β-amyloid plaques. (See *e.g.*, Davies, L. *et al.* (1988) *Neurology* 38:1688-1693) These observations suggest that β-AP deposition precedes, and contributes to, the destruction of neurons that occurs in AD. In further support of a direct pathogenic role for β-AP, β-amyloid has been shown to be toxic to mature neurons, both in culture and *in vivo*. Yankner, B.A. *et al.* (1989) *Science* 245:417-420; Yankner, B.A. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:9020-9023; Roher, A.E. *et al.* (1991) *Biochem. Biophys. Res. Commun.* 174:572-579; Kowall, N.W. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7247-7251. Furthermore, patients with hereditary cerebral hemorrhage with amyloidosis-Dutch-type (HCHWA-D), which is characterized by diffuse β-amyloid deposits within the cerebral cortex and cerebrovasculature, have been shown to have a point mutation that leads to an amino acid substitution within β-AP. Levy, E. *et al.* (1990) *Science* 248:1124-1126. This observation demonstrates that a specific alteration of the β-AP sequence can cause β-amyloid to be deposited.

Natural β-AP is derived by proteolysis from a much larger protein called the amyloid precursor protein (APP). Kang, J. *et al.* (1987) *Nature* 325:733; Goldgaber, D. *et al.* (1987) *Science* 235:877; Robakis, N.K. *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:4190; Tanzi, R.E. *et al.* (1987) *Science* 235:880. The APP gene maps to chromosome 21, thereby providing an explanation for the β-amyloid deposition seen at an early age in individuals with Down's syndrome, which is caused by trisomy of chromosome 21. Mann, D.M. *et al.* (1989) *Neuropathol. Appl. Neurobiol.* 15:317; Rumble, B. *et al.* (1989) *N. Eng. J. Med.* 320:1446. APP contains a single membrane spanning domain, with a long amino terminal region (about two-thirds of the protein) extending into the extracellular environment and a shorter carboxy-terminal region projecting into the cytoplasm. Differential splicing of the APP messenger RNA leads to at least five forms of APP, composed of either 563 amino acids (APP-563), 695 amino acids (APP-695), 714 amino acids (APP-714), 751 amino acids (APP-751) or 770 amino acids (APP-770).

Within APP, naturally-occurring β amyloid peptide begins at an aspartic acid residue at amino acid position 672 of APP-770. Naturally-occurring β-AP derived from proteolysis of APP is 39 to 43 amino acid residues in length, depending on the carboxy-terminal end point, which exhibits heterogeneity. The predominant circulating form of β-AP in the blood and cerebrospinal fluid of both AD patients and normal adults is β1-40 ("short β"). Seubert, P. *et al.* (1992) *Nature* 359:325; Shoji, M. *et al.* (1992) *Science* 258:126. However, β1-42 and β1-43 ("long β") also are forms in β-amyloid plaques. Masters, C. *et al.* (1985) *Proc. Natl. Acad. Sci. USA* 82:4245; Miller, D. *et al.* (1993) *Arch. Biochem. Biophys.* 301:41; Mori, H. *et al.* (1992) *J. Biol. Chem.* 267:17082. Although the precise molecular mechanism leading to β-APP aggregation and deposition is unknown, the process has been likened to that of nucleation-dependent polymerizations, such as protein crystallization, microtubule formation and actin polymerization. See *e.g.*, Jarrett, J.T. and Lansbury, P.T. (1993) *Cell* 73:1055-1058. In such processes, polymerization of monomer components does not occur until nucleus formation. Thus, these processes are characterized by a lag time before aggregation occurs, followed by rapid polymerization after nucleation. Nucleation can be accelerated by the addition of a "seed" or preformed nucleus, which results in rapid polymerization. The long β forms of β-AP have been shown to act as seeds, thereby accelerating polymerization of both long and short β-AP forms. Jarrett, J.T. *et al.* (1993) *Biochemistry* 32:4693.

In one study, in which amino acid substitutions were made in β-AP, two mutant β peptides were reported to interfere with polymerization of non-mutated β-AP when the mutant and non-mutant forms of peptide were mixed. Hilbich, C. *et al.* (1992) *J. Mol. Biol.* 228:460-473. Equimolar amounts of the mutant and non-mutant (*i.e.*, natural) β amyloid peptides were used to see this effect and the mutant peptides were reported to be unsuitable for use *in vivo*. Hilbich, C. *et al.* (1992), *supra*.

Summary of the Invention

This invention pertains to compounds, and pharmaceutical compositions thereof, that can bind to natural β amyloid peptides (β-AP), modulate the aggregation of natural β-AP and/or inhibit the neurotoxicity of natural β-APs. The compounds are modified in a manner which allows for increased biostability and prolonged elevated plasma levels. The β-amyloid modulator compounds of the invention comprise a peptidic structure, preferably based on β-amyloid peptide, that is composed entirely of D-amino acids. In various embodiments, the peptidic structure of the modulator compound comprises a D-amino acid sequence corresponding to a L-amino acid sequence found within natural β-AP, a D-amino acid sequence which is an inverso isomer of an L-amino acid sequence found within natural β-AP, a D-amino acid sequence which is a retro-inverso isomer of an L-amino acid sequence found within natural β-AP, or a D-amino acid sequence that is a scrambled or substituted version of an L-amino acid sequence found within natural β-AP. Preferably, the D-amino acid peptidic structure of the modulator is designed based upon a subregion of natural β-AP at positions 17-21 ($A\beta_{17-20}$ and $A\beta_{17-21}$, respectively), which has the amino acid sequences Leu-Val-Phe-Phe-Ala (SEQ ID NO:4). In preferred embodiments, a phenylalanine in the compounds of the invention is substituted with a phenylalanine analogue which is more stable and less prone to, for example, oxidative metabolism, or allows for increased brain levels of the compound.

In yet another embodiment, a modulator compound of the invention includes a β-amyloid peptide comprised of D-amino acids, L-amino acids or both, an inverso isomer of a β-amyloid peptide, or a retro-inverso isomer of a β-amyloid peptide which is attached to a hydrazine moiety, wherein the compound binds to natural β-amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

A modulator compound of the invention preferably comprises 3-20 D-amino acids, more preferably 3-10 D-amino acids and even more preferably 3-5 D-amino acids. The D-amino acid peptidic structure of the modulator can have free amino-, carboxy-, or carboxy amide- termini. Alternatively, the amino-terminus, the carboxy-terminus or both may be modified. For example, an N-terminal modifying group can be used that enhances the ability of the compound to inhibit Aβ aggregation. Moreover, the amino- and/or carboxy termini of the peptide can be modified to alter a pharmacokinetic property of the compound (such as stability, bioavailability, e.g., enhanced delivery of the compound across the blood brain barrier and entry into the brain, and the like). Preferred amino-terminal modifying groups include alkyl groups, e.g., methyl, ethyl, or isopropyl groups. Preferred carboxy-terminal modifying groups include amide groups, alkyl or aryl amide groups (*e.g.*, phenethylamide), hydroxy groups (*i.e.*, reduction products of peptide acids, resulting in peptide alcohols), acyl amide groups, and acetyl groups. Still further, a modulator compound can be modified to label the compound with a detectable substance (*e.g.*, a radioactive label).

In certain preferred embodiments, the invention provides a compound having the structure: N,N-dimethyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N,N-dimethyl-(D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N-methyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N-ethyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N-isopropyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Ala)-isopropylamide; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Ala)-dimethylamide; N,N-diethyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N,N-diethyl-(D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N,N-dimethyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N,N-dimethyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N,N-dimethyl-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; H-(Gly-D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N-ethyl-(Gly-D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N-ethyl-(Gly- D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N-methyl-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N-ethyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N-propyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N,N-diethyl-(Gly-D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; H-(D-Ile-D-Val-D-Phe-D-Phe-D-Ile)-NH_2; H-(D-Ile-D-Val-D-Phe-D-Phe-D-Ala-)-NH_2; H-(D-Ile- D-Ile-D-Phe-D-Phe- D-Ile)-NH_2; H-(D-Nle-D-Val-D-Phe-D-Phe-D-Ala-)-NH_2; H-(D-Nle-D-Val-D-Phe-D-Phe-D-Nle)-NH_2; 1-piperidine-acetyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; 1-piperidine-acetyl-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; H-D-Leu-D-Val-D-Phe-D-Phe-D-Leu-isopropylamide; H-D-Leu-D-Phe-D-Phe-D-Val-D-Leu-isopropylamide; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-methylamide; H-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-methylamide; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-OH; N-methyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-Cha-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-[*p*-F]Phe-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-[F_5]Phe-D-Leu)-NH_2; H-(D-Leu-D-Phe-D-Cha-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Phe- D-[*p*-F]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Phe- D-[F_5]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Phe-D-Lys-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Cha-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-[*p*-F]Phe-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-[F_5]Phe-D-Phe-D-Val-D-

Leu)-NH_2; H-(D-Leu- D-Lys-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Cha-D-Cha-D-Val-D-Leu)-NH_2; H-(D-Leu- D-[*p*-F]Phe-D-[*p*-F]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-[F_5]Phe-D-[F_5]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu- D-Lys- D-Lys-D-Val-D-Leu)-NH_2; N-methyl-(D-Leu-D-Val-D-Phe-D-Cha-D-Leu)-NH_2; N-methyl-(D-Leu-D-Val-D-Phe-D-[*p*-F]Phe-D-Leu)-NH_2; N-methyl-(D-Leu-D-Val-D-Phe-D-[F_5]Phe-D-Leu)-NH_2; H-D-Leu-D-Val-D-Phe-NH-(H-D-Leu-D-Val-D-Phe-)NH; H-D-Leu-D-Val-D-Phe-NH-NH-$COCH_3$; and H- D-Leu-D-Val-D-Phe-NH-NH_2.

Particularly preferred compounds of the invention are set forth in the Examples.

Another aspect of the invention pertains to pharmaceutical compositions. Typically, the pharmaceutical composition comprises a therapeutically effective amount of a modulator compound of the invention and a pharmaceutically acceptable carrier.

Yet another aspect of the invention pertains to methods for inhibiting aggregation of natural β-amyloid peptides. These methods comprise contacting the natural β-amyloid peptides with a modulator compound of the invention such that aggregation of the natural β-amyloid peptides is inhibited.

Yet another aspect of the invention pertains to methods for detecting the presence or absence of natural β-amyloid peptides in a biological sample. These methods comprise contacting a biological sample with a compound of the invention, wherein the compound is labeled with a detectable substance, and detecting the compound bound to natural β-amyloid peptides to thereby detect the presence or absence of natural β-amyloid peptides in the biological sample.

Still another aspect of the invention pertains to methods for treating a subject for a disorder associated with β-amyloidosis. These methods comprise administering to the subject a therapeutically effective amount of a modulator compound of the invention such that the subject is treated for a disorder associated with β-amyloidosis. Preferably, the disorder is Alzheimer's disease. Use of the modulators of the invention for therapy or for the manufacture of a medicament for the treatment of a disorder associated with β-amyloidosis is also encompassed by the invention.

Brief Description of the Drawings

Figure 1 is a table depicting the results from a brain uptake assay.

Figure 2 is a graph depicting the results from the fibril binding assay described in Example 2.

Detailed Description of the Invention

This invention pertains to compounds, and pharmaceutical compositions thereof, that can bind to natural β-amyloid peptides, modulate the aggregation of natural β amyloid peptides (β-AP) and/or inhibit the neurotoxicity of natural β-APs. The compounds are modified in a manner which allows for increased biostability and prolonged elevated plasma levels. A compound of the invention that modulates aggregation of natural β-AP, referred to herein interchangeably as a β amyloid modulator compound, a β amyloid modulator or simply a modulator, alters the aggregation of natural β-AP when the modulator is contacted with natural β-AP. Thus, a compound of the invention acts to alter the natural aggregation process or rate for β-AP, thereby disrupting this process. Preferably, the compounds inhibit β-AP aggregation. The compounds of the invention are characterized in that they comprise a peptidic structure composed entirely of D-amino acid residues. This peptidic structure is preferably based on β-amyloid peptide and can comprise, for example, a D-amino acid sequence corresponding to a L-amino acid sequence found within natural β-AP, a D-amino acid sequence which is an inverso isomer of an L-amino acid sequence found within natural β-AP, a D-amino acid sequence which is a retro-inverso isomer of an L-amino acid sequence found within natural β-AP, or a D-amino acid sequence that is a scrambled or substituted version of an L-amino acid sequence found within natural β-AP. In preferred embodiments, the phenylalanines in the compounds of the invention are substituted with phenylalanine analogues which are more stable and less prone to, for example, oxidative metabolism.

The invention encompasses modulator compounds comprising a D-amino acid peptidic structure having free amino-, carboxy-, or carboxy amide- termini, as well as modulator compounds in which the amino-terminus, the carboxy-terminus, and/or side chain(s) of the peptidic structure are modified.

The β amyloid modulator compounds of the invention can be selected based upon their ability to bind to natural β-amyloid peptides, modulate the aggregation of natural β-AP *in vitro* and/or inhibit the neurotoxicity of natural β-AP fibrils for cultured cells (using assays described herein, for example, the neurotoxicity assay, the nucleation assay, or the fibril binding assay). Preferred modulator compounds inhibit the aggregation of natural β-AP and/or inhibit the neurotoxicity of natural β-AP. However, modulator compounds selected based on one or both of these properties may have additional properties *in vivo* that may be beneficial in the treatment of amyloidosis (J. S. Pachter *et al.* (1998) "Aβ1-40 induced neurocytopathic activation of human monocytes is blocked by Aβ peptide aggregation inhibitors." *Neurobiology of Aging* (Abstracts: The 6th International Conference on Alzheimer's Disease and Related Disorders, Amsterdam, 18-23 July 1998) *19*, S128 (Abstract 540); R. Weltzein, A. *et al.* (1998) "Phagocytosis of Beta-Amyloid: A Possible Requisite for Neurotoxicity." *J. Neuroimmunology* (Special Issue: Abstracts of the International Society of Neuroimmunology Fifth International Congress, Montreal, Canada, 23-27 August 1998) **1998**, *90*, 32 (Abstract 162)). For example, the modulator compound may interfere with processing of natural β-AP (either by direct or indirect protease inhibition) or by modulation of processes that produce toxic β-AP, or other APP fragments, *in vivo*. Alternatively, modulator compounds may be selected based on these latter properties, rather than inhibition of Aβ aggregation *in vitro*. Moreover, modulator compounds of the invention that are selected based upon their interaction with natural β-AP also may interact with APP or with other APP fragments. Still further, a modulator compound of the invention can be characterized by its ability to bind to β-amyloid fibrils (which can be determined, for example, by radiolabeling the compound, contacting the compound with β-amyloid plaque and counting or detecting, e.g., by imaging, the compound bound to pathological forms of β-AP, e.g., the plaque), while not significantly altering the aggregation of the β-amyloid fibrils. Such a compound that binds efficiently to β-amyloid fibrils while not significantly altering the aggregation of the β-amyloid fibrils can be used, for example, to detect β-amyloid fibrils (*e.g.*, for diagnostic purposes, as described further herein). It should be appreciated, however, that the ability of a particular compound to bind to β-amyloid fibrils and/or modulate their aggregation may vary depending upon the concentration of the compound. Accordingly, a compound that, at a low concentration, binds to β-amyloid fibrils without altering their aggregation may nevertheless inhibit aggregation of the fibrils at a higher concentration. All such compounds having the property of binding to β-amyloid fibrils and/or modulating the aggregation of the fibrils are intended to be encompassed by the invention.

As used herein, a "modulator" of β-amyloid aggregation is intended to refer to an agent that, when contacted with natural β amyloid peptides, alters the aggregation of the natural β amyloid peptides. The term "aggregation of β amyloid peptides" refers to a process whereby the peptides associate with each other to form a multimeric, largely insoluble complex. The term "aggregation" further is intended to encompass β amyloid fibril formation and also encompasses β–amyloid plaques.

The terms "natural β-amyloid peptide", "natural β-AP" and "natural Aβ peptide", used interchangeably herein, are intended to encompass naturally occurring proteolytic cleavage products of the β amyloid precursor protein (APP) which are involved in β-AP aggregation and β-amyloidosis. These natural peptides include β-amyloid peptides having 39-43 amino acids (*i.e.*, $A\beta_{1-39}$, $A\beta_{1-40}$, $A\beta_{1-41}$, $A\beta_{1-42}$ and $A\beta_{1-43}$). The amino-terminal amino acid residue of natural β-AP corresponds to the aspartic acid residue at position 672 of the 770 amino acid residue form of the amyloid precursor protein ("APP-770"). The 43 amino acid long form of natural β-AP has the amino acid sequence DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT (also shown in SEQ ID NO:1), whereas the shorter forms have 1-4 amino acid residues deleted from the carboxy-terminal end. The amino acid sequence of APP-770 from position 672 (*i.e.*, the amino-terminus of natural β-AP) to its C-terminal end (103 amino acids) is shown in SEQ ID NO:2. The preferred form of natural β-AP for use in the aggregation assays described herein is $A\beta_{1-40}$ or $A\beta_{1-42}$.

In the presence of a modulator of the invention, aggregation of natural β amyloid peptides is "altered" or "modulated". The various forms of the term "alteration" or "modulation" are intended to encompass both inhibition of β-AP aggregation and promotion of β-AP aggregation. Aggregation of natural β-AP is "inhibited" in the presence of the modulator when there is a decrease in the amount and/or rate of β-AP aggregation as compared to the amount and/or rate of β-AP aggregation in the absence of the modulator. The various forms of the term "inhibition" are intended to include both complete and partial inhibition of β-AP aggregation. Inhibition of aggregation can be quantitated as the fold increase in the lag time for aggregation or as the decrease in the overall plateau level of aggregation (*i.e.*, total amount of aggregation), using an aggregation assay as described in the Examples. In various embodiments, a modulator of the invention increases the lag time of aggregation at least 1.2-fold, 1.5-fold, 1.8-fold, 2-fold, 2.5-fold, 3-fold, 4-fold or 5-fold, for example, when the compound is at a one molar equivalent to the β-AP. In various other embodiments, a modulator of the invention inhibits the plateau level of aggregation at least 10%, 20%, 30%, 40 %, 50 %, 75 % or 100 %.

A modulator which inhibits β-AP aggregation (an "inhibitory modulator compound") can be used to prevent or delay the onset of β-amyloid deposition. Preferably, inhibitory modulator compounds of the invention inhibit the formation and/or activity of neurotoxic aggregates of natural Aβ peptide (*i.e.*, the inhibitory compounds can be used to inhibit the neurotoxicity of β-AP). Additionally, the inhibitory compounds of the invention can reduce the neurotoxicity of preformed β-AP aggregates, indicating that the inhibitory modulators can either bind to preformed Aβ fibrils or soluble aggregate and modulate their inherent neurotoxicity or that the modulators can perturb the equilibrium between monomeric and aggregated forms of β-AP in favor of the non-neurotoxic form.

Alternatively, in another embodiment, a modulator compound of the invention promotes the aggregation of natural Aβ peptides. The various forms of the term "promotion" refer to an increase in the amount and/or rate of β-AP aggregation in the presence of the modulator, as compared to the amount and/or rate of β-AP aggregation in the absence of the modulator. Such a compound which promotes Aβ aggregation is referred to as a stimulatory modulator compound. Stimulatory modulator compounds may be useful for sequestering β-amyloid peptides, for example in a biological compartment where aggregation of β-AP may not be deleterious to thereby deplete β-AP from a biological compartment where aggregation of β-AP is deleterious. Moreover, stimulatory modulator compounds can be used to promote Aβ aggregation in *in vitro* aggregation assays (*e.g.*, assays such as those described in Example 2), for example in screening assays for test compounds that can then inhibit or reverse this Aβ aggregation (*i.e.*, a stimulatory modulator compound can act as a "seed" to promote the formation of Aβ aggregates).

In a preferred embodiment, the modulators of the invention are capable of altering β-AP aggregation when contacted with a molar excess amount of natural β-AP. A "molar excess amount of natural β-AP" refers to a concentration of natural β-AP, in moles, that is greater than the concentration, in moles, of the modulator. For example, if the modulator and β-AP are both present at a concentration of 1 μM, they are said to be "equimolar", whereas if the modulator is present at a concentration of 1 μM and the β-AP is present at a concentration of 5 μM, the β-AP is said to be present at a 5-fold molar excess amount compared to the modulator. In preferred embodiments, a modulator of the invention is effective at altering natural β-AP aggregation when the natural β-AP is present at at least a 2-fold, 3-fold or 5-fold molar excess compared to the concentration of the modulator. In other embodiments, the modulator is effective at altering β-AP aggregation when the natural β-AP is present at at least a 10-fold, 20-fold, 33-fold, 50-fold, 100-fold, 500-fold or 1000-fold molar excess compared to the concentration of the modulator.

As used herein, the term "β amyloid peptide comprised entirely of D-amino acids", as used in a modulator of the invention, is intended to encompass peptides having an amino acid sequence identical to that of the natural sequence in APP, as well as peptides having acceptable amino acid substitutions from the natural sequence, but which is composed of D-amino acids rather than the natural L-amino acids present in natural β-AP. Acceptable amino acid substitutions are those that do not affect and/or may improve the ability of the D-amino acid-containing peptide to alter natural β-AP aggregation. Moreover, particular amino acid substitutions may further contribute to the ability of the peptide to alter natural β-AP aggregation and/or may confer additional beneficial properties on the peptide (*e.g.*, increased solubility, reduced association with other amyloid proteins, etc.). A peptide having an identical amino acid sequence to that found within a parent peptide but in which all L-amino acids have been substituted with all D-amino acids is also referred to as an "inverso" compounds. For example, if a parent peptide is Thr-Ala-Tyr, the inverso form is D-Thr-D-Ala-D-Tyr.

As used herein, the term "retro-inverso isomer of a β amyloid peptide", as used in a modulator of the invention, is intended to encompass peptides in which the sequence of the amino acids is reversed as compared to the sequence in natural β-AP and all L-amino acids are replaced with D-amino acids. For example, if a parent peptide is Thr-Ala-Tyr, the retro-inverso form is D-Tyr-D-Ala-D-Thr. Compared to the parent peptide, a retro-inverso peptide has a reversed backbone while retaining substantially the original spatial conformation of the side chains, resulting in a retro-inverso isomer with a topology that closely resembles the parent peptide. See Goodman *et al.* "*Perspectives in Peptide Chemistry*" pp. 283-294 (1981). See also U.S. Patent No. 4,522,752 by Sisto for further description of "retro-inverso" peptides.

Various additional aspects of the modulators of the invention, and the uses thereof, are described in further detail in the following subsections.

I. Modulator Compounds

In one embodiment, a modulator compound of the invention comprises a β-amyloid peptide, the β-amyloid peptide being comprised entirely of D-amino acids, wherein the compound binds to natural β-amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. Preferably, the β-amyloid peptide of the modulator is comprised of 3-20 D-amino acids, more preferably 3-10 D-amino acids, and even more preferably 3-5 D-amino acids. In preferred embodiments, a phenylalanine in the compounds of the invention is substituted with a phenylalanine analogue which is more stable and less prone to, for example, oxidative metabolism.

In one embodiment, the β-amyloid peptide of the modulator is amino-terminally modified, for example, with a modifying group comprising an alkyl group such as a C1-C6 lower alkyl group, *e.g.*, a methyl, ethyl, or propyl group; or a cyclic, heterocyclic, polycyclic or branched alkyl group. Examples of suitable N-terminal modifying groups are described further in subsection II below. In another embodiment, the β-amyloid peptide of the modulator is carboxy-terminally modified, for example the modulator can comprise a peptide amide, a peptide alkyl or aryl amide (*e.g.*, a peptide phenethylamide) or a peptide alcohol. Examples of suitable C-terminal modifying groups are described further in subsections II and III below. The β-amyloid peptide of the modulator may be modified to enhance the ability of the modulator to alter β-AP aggregation or neurotoxicity. Additionally or alternatively, β-amyloid peptide of the modulator may be modified to alter a pharmacokinetic property of the modulator and/or to label the modulator with a detectable substance (described further in subsection III below).

In another embodiment, a modulator compound of the invention comprises a retro-inverso isomer of a β-amyloid peptide, wherein the compound binds to natural β-amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. Preferably, the retro-inverso isomer of the β-amyloid peptide is comprised of 3-20 D-amino acids, more preferably 3-10 D-amino acids, and even more preferably 3-5 D-amino acids. In preferred embodiments, the phenylalanines in the compounds of the invention are substituted with phenylalanine analogues which are more stable and less prone to, for example, oxidative metabolism.

In one embodiment, the retro-inverso isomer is amino-terminally modified, for example, with a modifying group comprising an alkyl group such as a C1-C6 lower alkyl group, *e.g.*, a methyl, ethyl, or propyl group; or a cyclic, heterocyclic, polycyclic or branched alkyl group. Examples of suitable N-terminal modifying groups are described further in subsection II below. In another embodiment, the retro-inverso isomer is carboxy-terminally modified, for example with an amide group, an alkyl or aryl amide group (*e.g.*, phenethylamide) or a hydroxy group (*i.e.*, the reduction product of a peptide acid, resulting in a peptide alcohol). Examples of suitable C-terminal modifying groups are described further in subsections II and III below. The retro-inverso isomer may be modified to enhance the ability of the modulator to alter β-AP aggregation or neurotoxicity. Additionally or alternatively, the retro-inverso isomer may be modified to alter a pharmacokinetic property of the modulator and/or to label the modulator with a detectable substance (described further in subsection III below).

In yet another embodiment, a modulator compound of the invention includes a β-amyloid peptide comprised entirely or partially of D-amino acids, an inverso isomer of a β-amyloid peptide, or a retro-inverso isomer of a β-amyloid peptide which is attached to a hydrazine moiety, wherein the compound binds to natural β-amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. Preferably, the modulator compound of the invention is comprised of 1-20 D-amino acids, more preferably 1-10 D-amino acids, even more preferably 1-5 D-amino acids, and most preferably 2-4 D-amino acids which are attached to a hydrazine moiety.

In one embodiment, the modulator compounds of the invention which include a hydrazine moiety are amino-terminally modified, for example with a modifying comprising an alkyl group, e.g., a methyl, ethyl, or isopropyl group. Examples of suitable N-terminal modifying groups are described further in subsection II below. In another embodiment, modulator compounds of the invention which include a hydrazine moiety are carboxy-terminally modified, for example with an acetyl. Examples of suitable C-terminal modifying groups are described further in subsections II and III below. The modulator compounds of the invention which include a hydrazine moiety may be modified to enhance the ability of the modulator to alter β-AP aggregation or neurotoxicity. Additionally or alternatively, the modulator compounds of the invention which include a hydrazine moiety may be modified to alter a pharmacokinetic property of the modulator and/or to label the modulator with a detectable substance (described further in subsection III below).

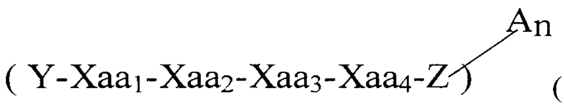
The modulators of the invention preferably are designed based upon the amino acid sequence of a subregion of natural β-AP. The term "subregion of a natural β-amyloid peptide" is intended to include amino-terminal and/or carboxy-terminal deletions of natural β-AP. The term "subregion of natural β-AP" is not intended to include full-length natural β-AP (*i.e.*, "subregion" does not include $A\beta_{1-39}$, $A\beta_{1-40}$, $A\beta_{1-41}$, $A\beta_{1-42}$ and $A\beta_{1-43}$). A preferred subregion of natural β-amyloid peptide is an "Aβ aggregation core domain" (ACD). As used herein, the term "Aβ aggregation core domain" refers to a subregion of a natural β-amyloid peptide that is sufficient to modulate aggregation of natural β-APs when this subregion, in its L-amino acid form, is appropriately modified (*e.g.*, modified at the amino-terminus), as described in detail in U.S. patent application Serial No. 08/548,998 and U.S. patent application Serial No. 08/616,081, the entire contents of each of which are expressly incorporated herein by reference. Preferably, the ACD is modeled after a subregion of natural β-AP that is less than 15 amino acids in length and more preferably is between 3-10 amino acids in length. In various embodiments, the ACD is modeled after a subregion of β-AP that is 10, 9, 8, 7, 6, 5, 4 or 3 amino acids in length. In one embodiment, the subregion of β-AP upon which the ACD is modeled is an internal or carboxy-terminal region of β-AP (*i.e.*, downstream of the amino-terminus at amino acid position 1). In another embodiment, the ACD is modeled after a subregion of β-AP that is hydrophobic. Preferred Aβ aggregation core domains encompass amino acid residues 17-20 or 17-21 of natural β-AP ($A\beta_{17-20}$ and $A\beta_{17-21}$, respectively) and analogues thereof, as described herein. The amino acid sequences of $A\beta_{17-20}$ and $A\beta_{17-21}$ are Leu-Val-Phe-Phe (SEQ ID NO:3) and Leu-Val-Phe-Phe-Ala (SEQ ID NO:4), respectively.

As demonstrated in the Examples, D-amino acid-containing modulators designed based upon the amino acid sequences of $A\beta_{17-20}$ and $A\beta_{17-21}$ are particularly effective inhibitors of Aβ aggregation and exhibit an enhanced biostability and prolonged elevated plasma levels. These modulators can comprise a D-amino acid sequence corresponding to the L-amino acid sequence of $A\beta_{17-20}$ or $A\beta_{17-21}$, a D-amino acid sequence which is an inverso isomer of the L-amino acid sequence of A$\beta_{17\text{-}20}$ or A$\beta_{17\text{-}21}$, a D-amino acid sequence which is a retro-inverso isomer of the L-amino acid sequence of A$\beta_{17\text{-}20}$ or A$\beta_{17\text{-}21}$, or a D-amino acid sequence that is a scrambled or substituted version of the L-amino acid sequence of A$\beta_{17\text{-}20}$ or A$\beta_{17\text{-}21}$. In preferred embodiments, a phenylalanine in the modulators designed based upon the amino acid sequences of A$\beta_{17\text{-}20}$ and A$\beta_{17\text{-}2}$ is substituted with a phenylalanine analogue which is more stable and less prone to, for example, oxidative metabolism. In other preferred embodiments, the modulators designed based upon the amino acid sequences of A$\beta_{17\text{-}20}$ and A$\beta_{17\text{-}2}$ further comprise a hydrazine moiety.

The D-amino acid-based modulators may have unmodified amino- and/or carboxy-termini and/or carboxy amide termini, or, alternatively, the amino-terminus, the carboxy-terminus, or both, may be modified (described further below). The peptidic structures of effective modulators generally are hydrophobic and are characterized by the presence of at least two D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure and a D-valine structure. As used herein, the term a "D-amino acid structure" (such as a "D-leucine structure", a "D-phenylalanine structure" or a "D-valine structure") is intended to include the D-amino acid, as well as analogues, derivatives and mimetics of the D-amino acid that maintain the functional activity of the compound (discussed further below). For example, the term "D-phenylalanine structure" is intended to include D-phenylalanine as well as D-cyclohexylalanine [D-cha], D-4-fluorophenylalanine (para-fluorophenylalanine) {[p-F]f or D-[p-F]Phe}, D-pentafluorophenylalanine {[F_5]f or D-[F_5]Phe}, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, D-pyridylalanine, D-homophenylalanine, methyltyrosine, and benzylserine, as well as substitution with D-lysine structure, D-valine structure, or a D-leucine structure. The term "D-leucine structure" is intended to include D-leucine, as well as substitution with D-valine, D-isoleucine, or other natural or non-natural amino acids having an aliphatic side chain, such as D-norleucine, or D-norvaline. The term "D-valine structure" is intended to include D-valine, as well as substitution with D-leucine or other natural or non-natural amino acid having an aliphatic side chain.

In other embodiments, the peptidic structure of the modulator comprises at least two D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure, a D-valine structure, a D-alanine structure, a D-tyrosine structure, a D-iodotyrosine structure, and a D-lysine structure. In another embodiment, the peptidic structure is comprised of at least three D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure and a D-valine structure. In yet another embodiment, the peptidic structure is comprised of at least three D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure, a D-valine structure, a D-alanine structure, a D-tyrosine structure, a D-iodotyrosine structure, and a D-lysine structure. In yet another embodiment, the peptidic structure comprises at least four D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure and a D-valine structure. In yet another embodiment, the peptidic structure is comprised of at least four D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure and a D-valine structure. In preferred embodiments, the peptidic structure includes at least one phenylalanine analogue which is more stable than phenylalanine and less prone to, for example, oxidative metabolism.

In one embodiment, the invention provides a β-amyloid modulator compound comprising a formula (I):



$$\left(Y\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Xaa_4\text{-}Z \right)\!\!\diagup\!A\,n \qquad (I)$$

wherein Xaa_1, Xaa_2, Xaa_3 and Xaa_4 are each D-amino acid structures and at least two of Xaa_1, Xaa_2, Xaa_3 and Xaa_4 are, independently, selected from the group consisting of a D-leucine structure, a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, and D-homophenylalanine, and a D-valine structure;

Y, which may or may not be present, is a structure having the formula $(Xaa)_a$, wherein Xaa is any D-amino acid structure and a is an integer from 1 to 15;

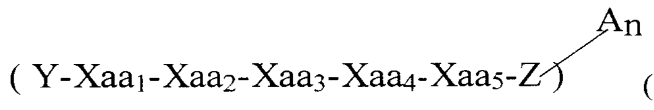
Z, which may or may not be present, is a structure having the formula $(Xaa)_b$, wherein Xaa is any D-amino acid structure and b is an integer from 1 to 15;

A, which may or may not be present, is a modifying group attached directly or indirectly to the compound; and

n is an integer from 1 to 15;

wherein Xaa_1, Xaa_2, Xaa_3, Xaa_4, Y, Z, A and n are selected such that the compound binds to natural β-amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides, and is less prone to metabolism, e.g., oxidative metabolism.

In a subembodiment of this formula, a fifth amino acid residue, Xaa_5, is specified C-terminal to Xaa_4 and Z, which may or may not be present, is a structure having the formula $(Xaa)_b$, wherein Xaa is any D-amino acid structure and b is an integer from 1 to 14. Accordingly, the invention provides a β-amyloid modulator compound comprising a formula (II):



$$\left(Y\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Xaa_4\text{-}Xaa_5\text{-}Z \diagup^{An} \right) \qquad \text{(II)}$$

wherein b is an integer from 1 to 14.

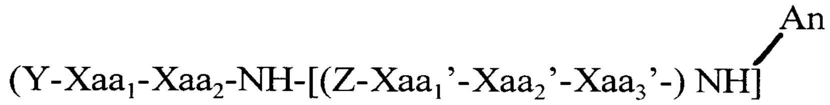
In a preferred embodiment, Xaa_1, Xaa_2, Xaa_3, Xaa_4 of formula (I) are selected based on the sequence of $A\beta_{17\text{-}20}$, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa_1 is a D-alanine structure or a D-leucine structure, Xaa_2 is a D-valine structure or a D-phenylalanine structure, Xaa_3 is a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, or a D-lysine structure and Xaa_4 is a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, or a D-lysine structure.

In another preferred embodiment, Xaa_1, Xaa_2, Xaa_3, Xaa_4 and Xaa_5 of formula (II) are selected based on the sequence of $A\beta_{17-21}$, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa_1 is a D-alanine structure or a D-leucine structure, Xaa_2 is a D-valine structure, Xaa_3 is a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, or a D-lysine structure, Xaa_4 is a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, D-pyridylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, or a D-lysine structure, and Xaa_5 is a D-alanine structure or a D-leucine structure.

In another preferred embodiment, Xaa_1, Xaa_2, Xaa_3 and Xaa_4 of formula (I) are selected based on the retro-inverso isomer of $A\beta_{17-20}$, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa_1 is a D-alanine structure, a D-leucine structure, or a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, a D-leucine structure, a D-valine structure, or a D-lysine structure; Xaa_2 is a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, D-pyridylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, or a D-lysine structure; Xaa_3 is a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, D-pyridylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, or a D-lysine structure; and Xaa_4 is a D-valine structure or a D-leucine structure.

In another preferred embodiment, Xaa_1, Xaa_2, Xaa_3, Xaa_4 and Xaa_5 of formula (II) are selected based on the retroinverso isomer of $A\beta_{17\text{-}21}$, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa_1 is a D-alanine structure, a D-leucine structure or a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, D-pyridylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, or a D-lysine structure; Xaa_2 is a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, D-pyridylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, or a D-lysine structure; Xaa_3 is a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, D-pyridylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, or a D-lysine structure; Xaa_4 is a D-valine structure or a D-leucine structure and Xaa_5 is a D-leucine structure.

In another embodiment, the invention provides a β-amyloid modulator compound comprising a formula (III):



$$(Y\text{-}Xaa_1\text{-}Xaa_2\text{-}NH\text{-}[(Z\text{-}Xaa_1'\text{-}Xaa_2'\text{-}Xaa_3'\text{-})\ NH]^{\diagup An} \qquad \text{(III)}$$

wherein Xaa_1 and Xaa_2 are each D-amino acid structures and at least two of Xaa_1 and Xaa_2 are, independently, selected from the group consisting of a D-leucine structure, a D-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, and D-homophenylalanine, a D-tyrosine structure, a D-iodotyrosine structure, a D-lysine structure, or a D-valine structure;

NH-NH is a hydrazine structure;

Y, which may or may not be present, is a structure having the formula $(Xaa)_a$, wherein Xaa is any D-amino acid structure and a is an integer from 1 to 15;

Xaa_1', Xaa_2', and Xaa_3' which may or may not be present, are each D-amino acid or L-amino acid structures and at least two of Xaa_1', Xaa_2', and Xaa_3'are, independently, selected from the group consisting of a D- or L-leucine structure, a D- or L-phenylalanine structure, e.g., D-cyclohexylalanine, D-4-fluorophenylalanine (para-fluorophenylalanine), D-pentafluorophenylalanine, chlorophenylalanine, bromophenylalanine, nitrophenylalanine, and D-homophenylalanine, a D- or L-tyrosine structure, a D- or L-iodotyrosine structure, a D- or L-lysine structure, or a D- or L-valine structure;

Z, which may or may not be present, is a structure having the formula $(Xaa)_b$, wherein Xaa is any D-amino acid structure and b is an integer from 1 to 15;

A, which may or may not be present, is a modifying group attached directly or indirectly to the compound; and

n is an integer from 1 to 15;

wherein Xaa_1, Xaa_2, Xaa_1', Xaa_2', Xaa_3', Y, Z, A and n are selected such that the compound binds to natural β-amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides, and is less prone to metabolism, e.g., oxidative metabolism.

In the modulators of the invention having the formula (I), (II), or (III) shown above, an optional modifying group ("A") is attached directly or indirectly to the peptidic structure of the modulator. (As used herein, the term "modulating group" and "modifying group" are used interchangeably to describe a chemical group directly or indirectly attached to a peptidic structure). For example, a modifying group(s) can be directly attached by covalent coupling to the peptidic structure or a modifying group(s) can be attached indirectly by a stable non-covalent association. In one embodiment of the invention, a modifying group is attached to the amino-terminus of the modulator. Alternatively, in another embodiment of the invention, a modifying group is attached to the carboxy-terminus of the modulator. In other embodiments, the modifying group is attached to both the amino and the carboxy-terminus of the modulator. In yet another embodiment, a modulating group(s) is attached to the side chain of at least one amino acid residues of the peptidic structure of the modulator (*e.g.*, through the epsilon amino group of a lysyl residue(s), through the carboxyl group of an aspartic acid residue(s) or a glutamic acid residue(s), through a hydroxy group of a tyrosyl residue(s), a serine residue(s) or a threonine residue(s) or other suitable reactive group on an amino acid side chain).

If a modifying group(s) is present, the modifying group is selected such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. Accordingly, since the β-AP peptide of the compound is modified from its natural state, the modifying group "A" as used herein is not intended to include hydrogen. In a modulator of the invention, a single modifying group may be attached to the peptidic structure or multiple modifying groups may be attached to the peptidic structure. The number of modifying groups is selected such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. However, n preferably is an integer between 1 and 60, more preferably between 1 and 30 and even more preferably between 1 and 10 or 1 and 5. In a preferred embodiment, A is an amino-terminal modifying group comprising a cyclic, heterocyclic, polycyclic, linear, or branched alkyl group and n=1. In another preferred embodiment, A is carboxy-terminally modifying group comprising an amide group, an alkyl amide group, an aryl amide group or a hydroxy group, and n=1. Suitable modifying groups are described further in subsections II and III below.

In preferred specific embodiments, the invention provides a β-amyloid modulator compound comprising a peptidic structure selected from the group consisting of (D-Leu-D-Val-D-Phe-D-Cha-D-Leu) (SEQ ID NO:5); (D-Leu-D-Val-D-Cha-D-Phe-D-Leu) (SEQ ID NO:6); (D-Leu-D-Val-D-Phe-D-[*p*-F]Phe-D-Leu) (SEQ ID NO:7); (D-Leu-D-Val-D-[*p*-F]Phe-D-Phe-D-Leu) (SEQ ID NO:8); (D-Leu-D-Val-D-Phe-D-[F-

$_5$]Phe-D-Leu) (SEQ ID NO:9); (D-Leu-D-Val-D-[F$_5$]Phe-D-Phe-D-Leu) (SEQ ID NO:10); (D-Leu-D-Phe-D-Cha-D-Val-D-Leu) (SEQ ID NO:11); (D-Leu-D-Phe-D-[*p*-F]Phe-D-Val-D-Leu) (SEQ ID NO:12); D-Leu-D-Phe-D-[F$_5$]Phe-D-Val-D-Leu) (SEQ ID NO:13); (D-Leu-D-Phe-D-Lys-D-Val-D-Leu) (SEQ ID NO:14); (D-Leu-D-Cha-D-Phe-D-Val-D-Leu) (SEQ ID NO:15); (D-Leu-D-[*p*-F]Phe-D-Phe-D-Val-D-Leu) (SEQ ID NO:16); (D-Leu-D-[F$_5$]Phe-D-Phe-D-Val-D-Leu) (SEQ ID NO:17); (D-Leu-D-Lys-D-Phe-D-Val-D-Leu) (SEQ ID NO:18); (D-Leu-D-Cha-D-Cha-D-Val-D-Leu) (SEQ ID NO:19); (D-Leu-D-Val-D-Cha-D-Cha-D-Leu) (SEQ ID NO:20); (D-Leu-D-[*p*-F]Phe-D-[*p*-F]Phe-D-Val-D-Leu) (SEQ ID NO:21); (D-Leu-D-Val-D-[*p*-F]Phe-D-[*p*-F]Phe-D-Leu) (SEQ ID NO:22); (D-Leu-D-[F$_5$]Phe-D-[F$_5$]Phe-D-Val-D-Leu) (SEQ ID NO:23); (D-Leu-D-Val-D-[F$_5$]Phe-D-[F$_5$]Phe-D-Leu) (SEQ ID NO:24); (D-Leu-D-Val-D-Phe) (SEQ D NO:25).

Any of the aforementioned specific peptidic structures can be amino-terminally and/or carboxy-terminally modified and described further in subsections II and/or III below.

Particularly preferred modulators of the invention include the following: N,N-dimethyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH$_2$; N,N-dimethyl-(D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH$_2$; N-methyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH$_2$; N-ethyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH$_2$; N-isopropyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH$_2$; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Ala)-isopropylamide; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Ala)-dimethylamide; N,N-diethyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH$_2$; N,N-diethyl-(D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH$_2$; N,N-dimethyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH$_2$; N,N-dimethyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH$_2$; N,N-dimethyl-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH$_2$; H-(Gly-D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH$_2$; N-ethyl-(Gly-D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH$_2$; N-ethyl-(Gly- D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH$_2$; N-methyl-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH$_2$; N-ethyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH$_2$; N-propyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH$_2$; N,N-diethyl-(Gly-D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH$_2$; H-(D-Ile-D-Val-D-Phe-D-Phe-D-Ile)-NH$_2$; H-(D-Ile-D-Val-D-Phe-D-Phe-D-Ala-)-NH$_2$; H-(D-Ile- D-Ile-D-Phe-D-Phe- D-Ile)-NH$_2$; H-(D-Nle-D-Val-D-Phe-D-Phe-D-Ala-)-NH$_2$; H-(D-Nle-D-Val-D-

Phe-D-Phe-D-Nle)-NH_2; 1-piperidine-acetyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; 1-piperidine-acetyl-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; H-D-Leu-D-Val-D-Phe-D-Phe-D-Leu-isopropylamide; H-D-Leu-D-Phe-D-Phe-D-Val-D-Leu-isopropylamide; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-methylamide; H-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-methylamide; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-OH; N-methyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-Cha-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-[*p*-F]Phe-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-[F_5]Phe-D-Leu)-NH_2; H-(D-Leu-D-Phe-D-Cha-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Phe- D-[*p*-F]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Phe- D-[F_5]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Phe-D-Lys-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Cha-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-[*p*-F]Phe-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-[F_5]Phe-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu- D-Lys-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Cha-D-Cha-D-Val-D-Leu)-NH_2; H-(D-Leu- D-[*p*-F]Phe-D-[*p*-F]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-[F_5]Phe-D-[F_5]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu- D-Lys- D-Lys-D-Val-D-Leu)-NH_2; N-methyl-(D-Leu-D-Val-D-Phe-D-Cha-D-Leu)-NH_2; N-methyl-(D-Leu-D-Val-D-Phe-D-[*p*-F]Phe-D-Leu)-NH_2; N-methyl-(D-Leu-D-Val-D-Phe-D-[F_5]Phe-D-Leu)-NH_2; H-D-Leu-D-Val-D-Phe-NH-(H-D-Leu-D-Val-D-Phe-)NH; H-D-Leu-D-Val-D-Phe-NH-NH-$COCH_3$; and H- D-Leu-D-Val-D-Phe-NH-NH_2.

Even more preferred compounds of the invention include PPI-1319: H-(D-Leu-D-Phe-[*p*-F]D-Phe-D-Val-D-Leu)-NH_2 and PPI:1019: N-methyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2. (As described above, D-Cha stands for D-cyclohexylalanine; [*p*-F]f or D-[p-F]Phe stands for D-4-fluorophenylalanine (also *para*-fluorophenylalanine); [F_5]f or D-[F_5]Phe stands for D-pentafluorophenylalanine; and D-Nle stands for D-norleucine).

The D-amino acid peptidic structures of the modulators of the invention are further intended to include other peptide modifications, including analogues, derivatives and mimetics, that retain the ability of the modulator to alter natural β-AP aggregation as described herein. For example, a D-amino acid peptidic structure of a modulator of the invention may be further modified to increase its stability, bioavailability, and solubility. The terms "analogue", "derivative" and "mimetic" as used herein are intended to include molecules which mimic the chemical structure of a D-peptidic structure and retain the functional properties of the D-peptidic structure. Approaches to designing peptide analogs, derivatives and mimetics are known in the art. For example, see Farmer, P.S. in Drug Design (E.J. Ariens, ed.) Academic Press, New York, 1980, vol. 10, pp. 119-143; Ball. J.B. and Alewood, P.F. (1990) *J. Mol. Recognition* 3:55; Morgan, B.A. and Gainor, J.A. (1989) *Ann. Rep. Med. Chem.* 24:243; and Freidinger, R.M. (1989) *Trends Pharmacol. Sci.* 10:270. See also Sawyer, T.K. (1995) "Peptidomimetic Design and Chemical Approaches to Peptide Metabolism" in Taylor, M.D. and Amidon, G.L. (eds.) *Peptide-Based Drug Design: Controlling Transport and Metabolism*, Chapter 17; Smith, A.B. 3rd, *et al.* (1995) *J. Am. Chem. Soc.* 117:11113-11123; Smith, A.B. 3rd, *et al.* (1994) *J. Am. Chem. Soc.* 116:9947-9962; and Hirschman, R., *et al.* (1993) *J. Am. Chem. Soc.* 115:12550-12568.

As used herein, a "derivative" of a compound X (*e.g.*, a peptide or amino acid) refers to a form of X in which one or more reaction groups on the compound have been derivatized with a substituent group. Examples of peptide derivatives include peptides in which an amino acid side chain, the peptide backbone, or the amino- or carboxy-terminus has been derivatized (*e.g.*, peptidic compounds with methylated amide linkages). As used herein an "analogue" of a compound X refers to a compound which retains chemical structures of X necessary for functional activity of X yet which also contains certain chemical structures which differ from X. An examples of an analogue of a naturally-occurring peptide is a peptide which includes one or more non-naturally-occurring amino acids. As used herein, a "mimetic" of a compound X refers to a compound in which chemical structures of X necessary for functional activity of X have been replaced with other chemical structures which mimic the conformation of X. Examples of peptidomimetics include peptidic compounds in which the peptide backbone is substituted with one or more benzodiazepine molecules (see *e.g.*, James, G.L. *et al.* (1993) *Science* 260:1937-1942).

Analogues of the modulator compounds of the invention are intended to include compounds in which one or more D-amino acids of the peptidic structure are substituted with a homologous amino acid such that the properties of the original modulator are maintained. Preferably conservative amino acid substitutions are made at one or more amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), β-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Non-limiting examples of homologous substitutions that can be made in the peptidic structures of the modulators of the invention include substitution of D-phenylalanine with D-tyrosine, D-pyridylalanine or D-homophenylalanine, substitution of D-leucine with D-valine or other natural or non-natural amino acid having an aliphatic side chain and/or substitution of D-valine with D-leucine or other natural or non-natural amino acid having an aliphatic side chain.

The term mimetic, and in particular, peptidomimetic, is intended to include isosteres. The term "isostere" as used herein is intended to include a chemical structure that can be substituted for a second chemical structure because the steric conformation of the first structure fits a binding site specific for the second structure. The term specifically includes peptide back-bone modifications (*i.e.*, amide bond mimetics) well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. Several peptide backbone modifications are known, including $\psi[CH_2S]$, $\psi[CH_2NH]$, $\psi[CSNH_2]$, $\psi[NHCO]$, $\psi[COCH_2]$, and ψ [(E) or (Z) CH=CH]. In the nomenclature used above, ψ indicates the absence of an amide bond. The structure that replaces the amide group is specified within the brackets.

Other possible modifications include an N-alkyl (or aryl) substitution (ψ [CONR]), or backbone crosslinking to construct lactams and other cyclic structures. Other derivatives of the modulator compounds of the invention include C-terminal hydroxymethyl derivatives, *O*-modified derivatives (*e.g.*, C-terminal hydroxymethyl benzyl ether), N-terminally modified derivatives including substituted amides such as alkylamides and hydrazides and compounds in which a C-terminal phenylalanine residue is replaced with a phenethylamide analogue (*e.g.*, Val-Phe-phenethylamide as an analogue of the tripeptide Val-Phe-Phe).

The modulator compounds of the invention can be incorporated into pharmaceutical compositions (described further in subsection V below) and can be used in detection and treatment methods as described further in subsection VI below.

II. Modifying Groups

In certain embodiments, the modulator compounds of the invention are coupled directly or indirectly to at least one modifying group (abbreviated as MG). The term "modifying group" is intended to include structures that are directly attached to the D-amino acid peptidic structure (*e.g.*, by covalent coupling), as well as those that are indirectly attached to the peptidic structure (*e.g.*, by a stable non-covalent association or by covalent coupling to additional amino acid residues, or mimetics, analogues or derivatives thereof, which may flank the Aβ-derived D-amino acid peptidic structure). For example, the modifying group can be coupled to the amino-terminus or carboxy-terminus of an Aβ-derived D-amino acid peptidic structure, or to a peptidic or peptidomimetic region flanking the core domain. Alternatively, the modifying group can be coupled to a side chain of at least one D-amino acid residue of an Aβ-derived D-amino acid peptidic structure, or to a peptidic or peptidomimetic region flanking the core domain (*e.g.*, through the epsilon amino group of a lysyl residue(s), through the carboxyl group of an aspartic acid residue(s) or a glutamic acid residue(s), through a hydroxy group of a tyrosyl residue(s), a serine residue(s) or a threonine residue(s) or other suitable reactive group on an amino acid side chain). Modifying groups covalently coupled to the D-amino acid peptidic structure can be attached by means and using methods well known in the art for linking chemical structures, including, for example, amide, alkylamino, carbamate, urea or ester bonds.

The term "modifying group" is intended to include groups that are not naturally coupled to natural Aβ peptides in their native form. Accordingly, the term "modifying group" is not intended to include hydrogen. The modifying group(s) is selected such that the modulator compound alters, and preferably inhibits, aggregation of natural β-amyloid peptides when contacted with the natural β-amyloid peptides or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides. Although not intending to be limited by mechanism, in embodiments where the modulator comprises a modifying group(s), the modifying group(s) is thought to function as a key pharmacophore that enhances the ability of the modulator to disrupt Aβ polymerization.

In a preferred embodiment, the modifying group(s) comprises an alkyl group. The term "alkyl", as used herein, refers to a straight or branched chain hydrocarbon group having from about 1 to about 10 carbon atoms. Exemplary alkyl groups include methyl, ethyl, dimethyl, diethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, and n-hexyl. An alkyl group may be unsubstituted, or may be substituted at one or more positions, with, e.g., halogens, alkyls, cycloalkyls, alkenyls, alkynyls, aryls, heterocycles, hydroxyls, aminos, nitros, thiols, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, $-CF_3$, -CN, or the like. Preferred alkyls are methyls, ethyls, dimethyls, diethyls, n-propyls, isopropyls.

In another embodiment, one modifying group, e.g., an alkyl group, is coupled to another modifying group. In yet another embodiment, a D-amino acid in a modulator compound of the invention is modified with two modifying groups. Accordingly, preferred modifying groups include a 1-piperidine acetyl group.

In a preferred embodiment, the modifying group(s) comprises a cyclic, heterocyclic, polycyclic or branched alkyl group. The term "cyclic group", as used herein, is intended to include cyclic saturated or unsaturated (*i.e.*, aromatic) group having from about 3 to 10, preferably about 4 to 8, and more preferably about 5 to 7, carbon atoms. Exemplary cyclic groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and cyclooctyl. Cyclic groups may be unsubstituted or substituted at one or more ring positions. Thus, a cyclic group may be substituted with, *e.g.*, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, aryls, heterocycles, hydroxyls, aminos, nitros, thiols amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, sulfonates, selenoethers, ketones, aldehydes, esters, $-CF_3$, -CN, or the like.

The term "heterocyclic group" is intended to include cyclic saturated or unsaturated (*i.e.*, aromatic) group having from about 3 to 10, preferably about 4 to 8, and more preferably about 5 to 7, carbon atoms, wherein the ring structure includes about one to four heteroatoms. Heterocyclic groups include pyrrolidine, oxolane, thiolane, imidazole, oxazole, piperidine, piperazine, morpholine and pyridine. The heterocyclic ring can be substituted at one or more positions with such substituents as, for example, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, aryls, other heterocycles, hydroxyl, amino, nitro, thiol, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, $-CF_3$, -CN, or the like. Heterocycles may also be bridged or fused to other cyclic groups as described below.

The term "polycyclic group" as used herein is intended to refer to two or more saturated or unsaturated (*i.e.*, aromatic) cyclic rings in which two or more carbons are common to two adjoining rings, *e.g.*, the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycyclic group can be substituted with such substituents as described above, as for example, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, hydroxyl, amino, nitro, thiol, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, $-CF_3$, -CN, or the like.

A preferred polycyclic group is a group containing a cis-decalin structure. Although not intending to be limited by mechanism, it is thought that the "bent" conformation conferred on a modifying group by the presence of a cis-decalin structure contributes to the efficacy of the modifying group in disrupting Aβ polymerization. Accordingly, other structures which mimic the "bent" configuration of the cis-decalin structure can also be used as modifying groups. An example of a cis-decalin containing structure that can be used as a modifying group is a cholanoyl structure, such as a cholyl group. For example, a modulator compound can be modified at its amino terminus with a cholyl group by reacting the aggregation core domain with cholic acid, a bile acid. Moreover, a modulator compound can be modified at its carboxy terminus with a cholyl group according to methods known in the art (see *e.g.*, Wess, G. *et al.* (1993) *Tetrahedron Letters*, 34:817-822; Wess, G. *et al.* (1992) *Tetrahedron Letters* 33:195-198; and Kramer, W. *et al.* (1992) *J. Biol. Chem.* 267:18598-18604). Cholyl derivatives and analogues can also be used as modifying groups. For example, a preferred cholyl derivative is Aic (3-(*O*-aminoethyl-*iso*)-cholyl), which has a free amino group that can be used to further modify the modulator compound (*e.g.*, a chelation group for ^{99m}Tc can be introduced through the free amino group of Aic). As used herein, the term "cholanoyl structure" is intended to include the cholyl group and derivatives and analogues thereof, in particular those which retain a four-ring cis-decalin configuration. Examples of cholanoyl structures include groups derived from other bile acids, such as deoxycholic acid, lithocholic acid, ursodeoxycholic acid, chenodeoxycholic acid and hyodeoxycholic acid, as well as other related structures such as cholanic acid, bufalin and resibufogenin (although the latter two compounds are not preferred for use as a modifying group). Another example of a *cis*-decalin containing compound is 5β-cholestan-3α-ol (the *cis*-decalin isomer of (+)-dihydrocholesterol). For further description of bile acid and steroid structure and nomenclature, see Nes, W.R. and McKean, M.L. *Biochemistry of Steroids and Other Isopentanoids*, University Park Press, Baltimore, MD, Chapter 2.

In addition to cis-decalin containing groups, other polycyclic groups may be used as modifying groups. For example, modifying groups derived from steroids or β-lactams may be suitable modifying groups. In one embodiment, the modifying group is a "biotinyl structure", which includes biotinyl groups and analogues and derivatives thereof (such as a 2-iminobiotinyl group). In another embodiment, the modifying group can comprise a "fluorescein-containing group", such as a group derived from reacting an Aβ-derived peptidic structure with 5-(and 6-)-carboxyfluorescein, succinimidyl ester or fluorescein isothiocyanate. In various other embodiments, the modifying group(s) can comprise an *N*-acetylneuraminyl group, a *trans*-4-cotininecarboxyl group, a 2-imino-1-imidazolidineacetyl group, an (*S*)-(-)-indoline-2-carboxyl group, a (-)-menthoxyacetyl group, a 2-norbornaneacetyl group, a γ-oxo-5-acenaphthenebutyryl, a (-)-2-oxo-4-thiazolidinecarboxyl group, a tetrahydro-3-furoyl group, a 2-iminobiotinyl group, a diethylenetriaminepentaacetyl group, a 4-morpholinecarbonyl group, a 2-thiopheneacetyl group or a 2-thiophenesulfonyl group.

In addition to the cyclic, heterocyclic and polycyclic groups discussed above, other types of modifying groups can be used in a modulator of the invention. For example, hydrophobic groups and branched alkyl groups may be suitable modifying groups. Examples include acetyl groups, phenylacetyl groups, phenylacetyl groups, diphenylacetyl groups, triphenylacetyl groups, isobutanoyl groups, 4-methylvaleryl groups, *trans*-cinnamoyl groups, butanoyl groups and 1-adamantanecarbonyl groups.

Yet another type of modifying group is a compound that contains a non-natural amino acid that acts as a beta-turn mimetic, such as a dibenzofuran-based amino acid described in Tsang, K.Y. et al. (1994) *J. Am. Chem. Soc.* 116:3988-4005; Diaz, H and Kelly, J.W. (1991) *Tetrahedron Letters* 41:5725-5728; and Diaz. H et al. (1992) *J. Am. Chem. Soc.* 114:8316-8318. An example of such a modifying group is a peptide-aminoethyldibenzofuranyl-proprionic acid (Adp) group (e.g., DDIIL-Adp) (SEQ ID NO: 31). This type of modifying group further can comprise one or more N-methyl peptide bonds to introduce additional steric hindrance to the aggregation of natural β-AP when compounds of this type interact with natural β-AP.

Yet another type of modifying group is an NH-OR group, where the R can be any of the modified or umodified alkyl or cycloalkyl groups described herein.

Non-limiting examples of suitable modifying groups, with their corresponding modifying reagents, are listed below:

Modifying Group	Modifying Reagent
Methyl-	Methylamine, Fmoc-D-[Me]-Leu-OH,methylamine and a bromoacetylpeptide
Ethyl-	Ethylamine, acetaldehyde and sodium cyanoborohydride, ethylamine and a bromoacetylpeptide
Propyl-	Propylamine, propionaldehyde and sodium cyanoborohydride, propylamine and a bromoacetylpeptide
Isopropyl-	Isopropylamine, isopropylamine and a bromoacetylpeptide
Piperidine-	Piperidine and a bromoacetylpeptide
Acetyl-	Acetic anhydride, acetic acid
Dimethyl-	Methylamine, formaldehyde and sodium cyanoborohydride
Diethyl-	Acetaldehyde and sodium cyanoborohydride
Cholyl-	Cholic acid
Lithocholyl-	Lithocholic acid
Hyodeoxycholyl-	Hyodeoxycholic acid
Chenodeoxycholyl-	Chenodeoxycholic acid
Ursodeoxycholyl-	Ursodeoxycholic acid
3-Hydroxycinnamoyl-	3-Hydroxycinnamic acid
4-Hydroxycinnamoyl-	4-Hydroxycinnamic acid
2-Hydroxycinnamoyl-	2-Hydroxycinnamic acid
3-Hydroxy-4-methoxycinnamoyl-	3-Hydroxy-4-methoxycinnamic acid
4-Hydroxy-3-methoxycinnamoyl-	4-Hydroxy-3-methoxycinnamic acid
2-Carboxycinnamoyl-	2-Carboxycinnamic acid

3-Formylbenzoyl	3-Carboxybenzaldehyde
4-Formylbenzoyl	4-Carboxybenzaldehyde
3,4,-Dihydroxyhydrocinnamoyl-	3,4,-Dihydroxyhydrocinnamic acid
3,7-Dihydroxy-2-napthoyl-	3,7-Dihydroxy-2-naphthoic acid
4-Formylcinnamoyl-	4-Formylcinnamic acid
2-Formylphenoxyacetyl-	2-Formylphenoxyacetic acid
8-Formyl-1-napthoyl	1,8-napthaldehydic acid
4-(hydroxymethyl)benzoyl-	4-(hydroxymethyl)benzoic acid
4-Hydroxyphenylacetyl-	4-Hydroxyphenylacetic acid
3-Hydroxybenzoyl-	3-Hydroxybenzoic acid
4-Hydroxybenzoyl-	4-Hydroxybenzoic acid
5-Hydantoinacetyl-	5-Hydantoinacetic acid
L-Hydroorotyl-	L-Hydroorotic acid
4-Methylvaleryl-	4-Methylvaleric acid
2,4-Dihydroxybenzoyl-	2,4-Dihydroxybenzoic acid
3,4-Dihydroxycinnamoyl-	3,4-Dihydroxycinnamic acid
3,5-Dihydroxy-2-naphthoyl-	3,5-Dihydroxy-2-naphthoic acid
3-Benzoylpropanoyl-	3-Benzoylpropanoic acid
trans-Cinnamoyl-	*trans*-Cinnamic acid
Phenylacetyl-	Phenylacetic acid
Diphenylacetyl-	Diphenylacetic acid
Triphenylacetyl-	Triphenylacetic acid
2-Hydroxyphenylacetyl-	2-Hydroxyphenylacetic acid
3-Hydroxyphenylacetyl-	3-Hydroxyphenylacetic acid
4-Hydroxyphenylacetyl-	4-Hydroxyphenylacetic acid
(±)-Mandelyl-	(±)-Mandelic acid
(±)-2,4-Dihydroxy-3,3-dimethylbutanoyl	(±)-Pantolactone
Butanoyl-	Butanoic anhydride
Isobutanoyl-	Isobutanoic anhydride
Hexanoyl-	Hexanoic anhydride

Propionyl-	Propionic anhydride
3-Hydroxybutyroyl	β-Butyrolactone
4-Hydroxybutyroyl	γ-Butyrolactone
3-Hydroxypropionoyl	β-Propiolactone
2,4-Dihydroxybutyroyl	α-Hydroxy-β-Butyrolactone
1-Adamantanecarbonyl-	1-Adamantanecarbonic acid
Glycolyl-	Glycolic acid
DL-3-(4-hydroxyphenyl)lactyl-	DL-3-(4-hydroxyphenyl)lactic acid
3-(2-Hydroxyphenyl)propionyl-	3-(2-Hydroxyphenyl)propionic acid
4-(2-Hydroxyphenyl)propionyl-	4-(2-Hydroxyphenyl)propionic acid
D-3-Phenyllactyl-	D-3-Phenyllactic acid
Hydrocinnamoyl-	Hydrocinnamic acid
3-(4-Hydroxyphenyl)propionyl-	3-(4-Hydroxyphenyl)propionic acid
L-3-Phenyllactyl-	L-3-Phenyllactic acid
4-methylvaleryl	4-methylvaleric acid
3-pyridylacetyl	3-pyridylacetic acid
4-pyridylacetyl	4-pyridylacetic acid
Isonicotinoyl	
4-quinolinecarboxyl	4-quinolinecarboxylic acid
1-isoquinolinecarboxyl	1-isoquinolinecarboxylic acid
3-isoquinolinecarboxyl	3-isoquinolinecarboxylic acid

Preferred modifying groups include methyl-containing groups, ethyl-containing groups, propyl-containing groups, and piperidine-containing groups, e.g., a 1-piperidine-acetyl group.

III. Additional Chemical Modifications of Aβ Modulators

A β-amyloid modulator compound of the invention can be further modified to alter the specific properties of the compound while retaining the ability of the compound to alter Aβ aggregation and inhibit Aβ neurotoxicity. For example, in one embodiment, the compound is further modified to alter a pharmacokinetic property of the compound, such as *in vivo* stability or half-life. In another embodiment, the compound is further modified to label the compound with a detectable substance. In yet another embodiment, the compound is further modified to couple the compound to an additional therapeutic moiety. Schematically, a modulator of the invention comprising a D-amino acid Aβ aggregation core domain coupled directly or indirectly to at least one modifying group can be illustrated as MG-ACD, whereas this compound which has been further modified to alter the properties of the modulator can be illustrated as MG-ACD-CM, wherein CM represents an additional chemical modification.

To further chemically modify the compound, such as to alter the pharmacokinetic properties of the compound, reactive groups can be derivatized. For example, when the modifying group is attached to the amino-terminal end of the aggregation core domain, the carboxy-terminal end of the compound can be further modified. Preferred C-terminal modifications include those which reduce the ability of the compound to act as a substrate for carboxypeptidases. Examples of preferred C-terminal modifiers include an amide group (*i.e.*, a peptide amide), an alkyl or aryl amide group (*e.g.*, an ethylamide group or a phenethylamide group) a hydroxy group (*i.e.*, a peptide alcohol) and various non-natural amino acids, such as D-amino acids and β-alanine. Alternatively, when the modifying group is attached to the carboxy-terminal end of the aggregation core domain, the amino-terminal end of the compound can be further modified, for example, to reduce the ability of the compound to act as a substrate for aminopeptidases.

A modulator compound can be further modified to label the compound by reacting the compound with a detectable substance. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include ^{14}C, ^{123}I, ^{124}I, ^{125}I, ^{131}I, ^{99m}Tc, ^{35}S or ^{3}H. In a preferred embodiment, a modulator compound is radioactively labeled with ^{14}C, either by incorporation of ^{14}C into the modifying group or one or more amino acid structures in the modulator compound. Labeled modulator compounds can be used to assess the *in vivo* pharmacokinetics of the compounds, as well as to detect Aβ aggregation, for example for diagnostic purposes. Aβ aggregation can be detected using a labeled modulator compound either *in vivo* or in an *in vitro* sample derived from a subject.

Preferably, for use as an *in vivo* diagnostic agent, a modulator compound of the invention is labeled with radioactive technetium or iodine. Accordingly, in one embodiment, the invention provides a modulator compound labeled with technetium, preferably ^{99m}Tc. Methods for labeling peptide compounds with technetium are known in the art (see *e.g.*, U.S. Patent Nos. 5,443,815, 5,225,180 and 5,405,597, all by Dean *et al.*; Stepniak-Biniakiewicz, D., *et al.* (1992) *J. Med. Chem.* 35:274-279; Fritzberg, A.R., *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4025-4029; Baidoo, K.E., *et al.* (1990) *Cancer Res. Suppl.* 50:799s-803s; and Regan, L. and Smith, C.K. (1995) *Science* 270:980-982). A modifying group can be chosen that provides a site at which a chelation group for ^{99m}Tc can be introduced, such as the Aic derivative of cholic acid, which has a free amino group. In another embodiment, the invention provides a modulator compound labeled with radioactive iodine. For example, a phenylalanine residue within the Aβ sequence (such as Phe_{19} or Phe_{20}) can be substituted with radioactive iodotyrosyl. Any of the various isotopes of radioactive iodine can be incorporated to create a diagnostic agent. Preferably, ^{123}I (half-life = 13.2 hours) is used for whole body scintigraphy, ^{124}I (half life = 4 days) is used for positron emission tomography (PET), ^{125}I (half life = 60 days) is used for metabolic turnover studies and ^{131}I (half life = 8 days) is used for whole body counting and delayed low resolution imaging studies.

Furthermore, an additional modification of a modulator compound of the invention can serve to confer an additional therapeutic property on the compound. That is, the additional chemical modification can comprise an additional functional moiety. For example, a functional moiety which serves to break down or dissolve amyloid plaques can be coupled to the modulator compound. In this form, the MG-ACD portion of the modulator serves to target the compound to Aβ peptides and disrupt the polymerization of the Aβ peptides, whereas the additional functional moiety serves to break down or dissolve amyloid plaques after the compound has been targeted to these sites.

In an alternative chemical modification, a β-amyloid compound of the invention is prepared in a "prodrug" form, wherein the compound itself does not modulate Aβ aggregation, but rather is capable of being transformed, upon metabolism *in vivo*, into a β-amyloid modulator compound as defined herein. For example, in this type of compound, the modulating group can be present in a prodrug form that is capable of being converted upon metabolism into the form of an active modulating group. Such a prodrug form of a modifying group is referred to herein as a "secondary modifying group." A variety of strategies are known in the art for preparing peptide prodrugs that limit metabolism in order to optimize delivery of the active form of the peptide-based drug (see *e.g.*, Moss, J. (1995) in *Peptide-Based Drug Design: Controlling Transport and Metabolism*, Taylor, M.D. and Amidon, G.L. (eds), Chapter 18. Additionally strategies have been specifically tailored to achieving CNS delivery based on "sequential metabolism" (see *e.g.*, Bodor, N., *et al.* (1992) *Science* 257:1698-1700; Prokai, L., *et al.* (1994) *J. Am. Chem. Soc.* 116:2643-2644; Bodor, N. and Prokai, L. (1995) in *Peptide-Based Drug Design: Controlling Transport and Metabolism*, Taylor, M.D. and Amidon, G.L. (eds), Chapter 14. In one embodiment of a prodrug form of a modulator of the invention, the modifying group comprises an alkyl ester to facilitate blood-brain barrier permeability.

Modulator compounds of the invention can be prepared by standard techniques known in the art. The peptide component of a modulator can be synthesized using standard techniques such as those described in Bodansky, M. *Principles of Peptide*

Synthesis, Springer Verlag, Berlin (1993) and Grant, G.A (ed.). *Synthetic Peptides: A User's Guide*, W.H. Freeman and Company, New York (1992). Automated peptide synthesizers are commercially available (*e.g.*, Advanced ChemTech Model 396; Milligen/ Biosearch 9600). Additionally, one or more modulating groups can be attached to the Aβ-derived peptidic component (*e.g.*, an Aβ aggregation core domain) by standard methods, for example using methods for reaction through an amino group (*e.g.*, the alpha-amino group at the amino-terminus of a peptide), a carboxyl group (*e.g.*, at the carboxy terminus of a peptide), a hydroxyl group (*e.g.*, on a tyrosine, serine or threonine residue) or other suitable reactive group on an amino acid side chain (see *e.g.*, Greene, T.W and Wuts, P.G.M. *Protective_Groups in Organic Synthesis*, John Wiley and Sons, Inc., New York (1991). Exemplary syntheses of D-amino acid β amyloid modulator are described further in Example 1.

IV. Screening Assays

Another aspect of the invention pertains to a method for selecting a modulator of β-amyloid aggregation. In the method, a test compound is contacted with natural β amyloid peptides, the aggregation of the natural β-AP is measured and a modulator is selected based on the ability of the test compound to alter the aggregation of the natural β-AP (*e.g.*, inhibit or promote aggregation). In a preferred embodiment, the test compound is contacted with a molar excess amount of the natural β-AP. The amount and/or rate of natural β-AP aggregation in the presence of the test compound can be determined by a suitable assay indicative of β-AP aggregation, as described herein (see *e.g.*, Example 2).

In a preferred assay, the natural β-AP is dissolved in solution in the presence of the test compound and aggregation of the natural β-AP is assessed in a nucleation assay (see Example 2) by assessing the turbidity of the solution over time, as measured by the apparent absorbance of the solution at 405 nm (described further in Example 2; see also Jarrett *et al.* (1993) *Biochemistry* 32:4693-4697). In the absence of a β-amyloid modulator, the A_{405nm} of the solution typically stays relatively constant during a lag time in which the β-AP remains in solution, but then the A_{405nm} of the solution rapidly increases as the β-AP aggregates and comes out of solution, ultimately reaching a plateau level (*i.e.*, the A_{405nm} of the solution exhibits sigmoidal kinetics over time). In contrast, in the presence of a test compound that inhibits β-AP aggregation, the A_{405nm} of the solution is reduced compared to when the modulator is absent. Thus, in the presence of the inhibitory modulator, the solution may exhibit an increased lag time, a decreased slope of aggregation and/or a lower plateau level compared to when the modulator is absent. This method for selecting a modulator of β-amyloid polymerization can similarly be used to select modulators that promote β-AP aggregation. Thus, in the presence of a modulator that promotes β-AP aggregation, the A_{405nm} of the solution is increased compared to when the modulator is absent (*e.g.*, the solution may exhibit an decreased lag time, increase slope of aggregation and/or a higher plateau level compared to when the modulator is absent).

Another assay suitable for use in the screening method of the invention, a seeded extension assay, is also described further in Example 2. In this assay, β-AP monomer and an aggregated β-AP "seed" are combined, in the presence and absence of a test compound, and the amount of β-fibril formation is assayed based on enhanced emission of the dye Thioflavine T when contacted with β-AP fibrils. Moreover, β-AP aggregation can be assessed by electron microscopy (EM) of the β-AP preparation in the presence or absence of the modulator. For example, β amyloid fibril formation, which is detectable by EM, is reduced in the presence of a modulator that inhibits β-AP aggregation (*i.e.*, there is a reduced amount or number of β-fibrils in the presence of the modulator), whereas β fibril formation is increased in the presence of a modulator that promotes β-AP aggregation (*i.e.*, there is an increased amount or number of β-fibrils in the presence of the modulator).

Another preferred assay for use in the screening method of the invention to select suitable modulators is the neurotoxicity assay described in Example 3. Compounds are selected which inhibit the formation of neurotoxic Aβ aggregates and/or which inhibit the neurotoxicity of preformed Aβ fibrils. This neurotoxicity assay is considered to be predictive of neurotoxicity *in vivo*. Accordingly, inhibitory activity of a modulator compound in the *in vitro* neurotoxicity assay is predictive of similar inhibitory activity of the compound for neurotoxicity *in vivo*.

V. Pharmaceutical Compositions

Another aspect of the invention pertains to pharmaceutical compositions of the β-amyloid modulator compounds of the invention. In one embodiment, the composition includes a β amyloid modulator compound in a therapeutically or prophylactically effective amount sufficient to alter, and preferably inhibit, aggregation of natural β-amyloid peptides, and a pharmaceutically acceptable carrier. In another embodiment, the composition includes a β amyloid modulator compound in a therapeutically or prophylactically effective amount sufficient to inhibit the neurotoxicity of natural β-amyloid peptides, and a pharmaceutically acceptable carrier. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as reduction or reversal or β-amyloid deposition and/or reduction or reversal of Aβ neurotoxicity. A therapeutically effective amount of modulator may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the modulator to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the modulator are outweighed by the therapeutically beneficial effects. The potential neurotoxicity of the modulators of the invention can be assayed using the cell-based assay described in Example 6 and a therapeutically effective modulator can be selected which does not exhibit significant neurotoxicity. In a preferred embodiment, a therapeutically effective amount of a modulator is sufficient to alter, and preferably inhibit, aggregation of a molar excess amount of natural β-amyloid peptides. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as preventing or inhibiting the rate of β-amyloid deposition and/or Aβ neurotoxicity in a subject predisposed to β-amyloid deposition. A prophylactically effective amount can be determined as described above for the therapeutically effective amount. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

One factor that may be considered when determining a therapeutically or prophylactically effective amount of a β amyloid modulator is the concentration of natural β-AP in a biological compartment of a subject, such as in the cerebrospinal fluid (CSF) of the subject. The concentration of natural β-AP in the CSF has been estimated at 3 nM (Schwartzman, (1994) *Proc. Natl. Acad. Sci. USA* 91:8368-8372). A non-limiting range for a therapeutically or prophylactically effective amounts of a β amyloid modulator is 0.01 nM-10 μM. It is to be noted that dosage values may vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

The amount of active compound in the composition may vary according to factors such as the disease state, age, sex, and weight of the individual, each of which may affect the amount of natural β-AP in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. In one embodiment, the carrier is suitable for parenteral administration. Preferably, the carrier is suitable for administration into the central nervous system (*e.g.*, intraspinally or intracerebrally). Alternatively, the carrier can be suitable for intravenous, intraperitoneal or intramuscular administration. In another embodiment, the carrier is suitable for oral administration. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. Moreover, the modulators can be administered in a time release formulation, for example in a composition which includes a slow release polymer. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic copolymers (PLG). Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, β-amyloid modulator) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

A modulator compound of the invention can be formulated with one or more additional compounds that enhance the solubility of the modulator compound. Preferred compounds to be added to formulations to enhance the solubility of the modulators are cyclodextrin derivatives, preferably hydroxypropyl-γ-cyclodextrin. Drug delivery vehicles containing a cyclodextrin derivative for delivery of peptides to the central nervous system are described in Bodor, N., *et al.* (1992) *Science* 257:1698-1700. For the β-amyloid modulators described herein, inclusion in the formulation of hydroxypropyl-γ-cyclodextrin at a concentration 50-200 mM increases the aqueous solubility of the compounds. In addition to increased solubility, inclusion of a cyclodextrin derivative in the formulation may have other beneficial effects, since β-cyclodextrin itself has been reported to interact with the Aβ peptide and inhibit fibril formation *in vitro* (Camilleri, P., *et al.* (1994) *FEBS Letters* 341:256-258. Accordingly, use of a modulator compound of the invention in combination with a cyclodextrin derivative may result in greater inhibition of Aβ aggregation than use of the modulator alone. Chemical modifications of cyclodextrins are known in the art (Hanessian, S., *et al.* (1995) J. Org. Chem. 60:4786-4797). In addition to use as an additive in a pharmaceutical composition containing a modulator of the invention, cyclodextrin derivatives may also be useful as modifying groups and, accordingly, may also be covalently coupled to an Aβ peptide compound to form a modulator compound of the invention.

Another preferred formulation for the modulator compounds to enhance brain uptake comprises the detergent Tween-80, polyethylene glycol (PEG) and ethanol in a saline solution. A non-limiting example of such a preferred formulation is 0.16% Tween-80, 1.3% PEG-3000 and 2% ethanol in saline.

In another embodiment, a pharmaceutical composition comprising a modulator of the invention is formulated such that the modulator is transported across the blood-brain barrier (BBB). Various strategies known in the art for increasing transport across the BBB can be adapted to the modulators of the invention to thereby enhance transport of the modulators across the BBB (for reviews of such strategies, see *e.g.*, Pardridge, W.M. (1994) *Trends in Biotechnol.* 12:239-245; Van Bree, J.B. *et al.* (1993) *Pharm. World Sci.* 15:2-9; and Pardridge, W.M. *et al.* (1992) *Pharmacol. Toxicol.* 71:3-10). In one approach, the modulator is chemically modified to form a prodrug with enhanced transmembrane transport. Suitable chemical modifications include covalent linking of a fatty acid to the modulator through an amide or ester linkage (see *e.g.*, U.S. Patent 4,933,324 and PCT Publication WO 89/07938, both by Shashoua; U.S. Patent 5,284,876 by Hesse *et al.*; Toth, I. *et al.* (1994) *J. Drug Target.* 2:217-239; and Shashoua, V.E. *et al.* (1984) *J. Med. Chem.* 27:659-664) and glycating the modulator (see *e.g.*, U.S. Patent 5,260,308 by Poduslo *et al.*). Also, N-acylamino acid derivatives may be used in a modulator to form a "lipidic" prodrug (see *e.g.*, 5,112,863 by Hashimoto *et al.*).

In another approach for enhancing transport across the BBB, a peptidic or peptidomimetic modulator is conjugated to a second peptide or protein, thereby forming a chimeric protein, wherein the second peptide or protein undergoes absorptive-mediated or receptor-mediated transcytosis through the BBB. Accordingly, by coupling the modulator to this second peptide or protein, the chimeric protein is transported across the BBB. The second peptide or protein can be a ligand for a brain capillary endothelial cell receptor ligand. For example, a preferred ligand is a monoclonal antibody that specifically binds to the transferrin receptor on brain capillary endothelial cells (see *e.g.*, U.S. Patents 5,182,107 and 5,154,924 and PCT Publications WO 93/10819 and WO 95/02421, all by Friden *et al.*). Other suitable peptides or proteins that can mediate transport across the BBB include histones (see *e.g.*, U.S. Patent 4,902,505 by Pardridge and Schimmel) and ligands such as biotin, folate, niacin, pantothenic acid, riboflavin, thiamin, pryridoxal and ascorbic acid (see *e.g.*, U.S. Patents 5,416,016 and 5,108,921, both by Heinstein). Additionally, the glucose transporter GLUT-1 has been reported to transport glycopeptides (L-serinyl-β-D-glucoside analogues of [Met5]enkephalin) across the BBB (Polt, R. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:7114-1778). Accordingly, a modulator compound can be coupled to such a glycopeptide to target the modulator to the GLUT-1 glucose transporter. For example, a modulator compound which is modified at its amino terminus with the modifying group Aic (3-(*O*-aminoethyl-*iso*)-cholyl, a derivative of cholic acid having a free amino group) can be coupled to a glycopeptide through the amino group of Aic by standard methods. Chimeric proteins can be formed by recombinant DNA methods (*e.g.*, by formation of a chimeric gene encoding a fusion protein) or by chemical crosslinking of the modulator to the second peptide or protein to form a chimeric protein. Numerous chemical crosslinking agents are known in the (*e.g.*, commercially available from Pierce, Rockford IL). A crosslinking agent can be chosen which allows for high yield coupling of the modulator to the second peptide or protein and for subsequent cleavage of the linker to release bioactive modulator. For example, a biotin-avidin-based linker system may be used.

In yet another approach for enhancing transport across the BBB, the modulator is encapsulated in a carrier vector which mediates transport across the BBB. For example, the modulator can be encapsulated in a liposome, such as a positively charged unilamellar liposome (see *e.g.*, PCT Publications WO 88/07851 and WO 88/07852, both by Faden) or in polymeric microspheres (see *e.g.*, U.S. Patent 5,413,797 by Khan *et al.*, U.S. Patent 5,271,961 by Mathiowitz *et al.* and 5,019,400 by Gombotz *et al.*). Moreover, the carrier vector can be modified to target it for transport across the BBB. For example, the carrier vector (*e.g.*, liposome) can be covalently modified with a molecule which is actively transported across the BBB or with a ligand for brain endothelial cell receptors, such as a monoclonal antibody that specifically binds to transferrin receptors (see *e.g.*, PCT Publications WO 91/04014 by Collins *et al.* and WO 94/02178 by Greig *et al.*).

In still another approach to enhancing transport of the modulator across the BBB, the modulator is coadministered with another agent which functions to permeabilize the BBB. Examples of such BBB "permeabilizers" include bradykinin and bradykinin agonists (see *e.g.*, U.S. Patent 5,112,596 by Malfroy-Camine) and peptidic compounds disclosed in U.S. Patent 5,268,164 by Kozarich *et al.*

Assays that measure the *in vitro* stability of the modulator compounds in cerebrospinal fluid (CSF) and the degree of brain uptake of the modulator compounds in animal models can be used as predictors of in vivo efficacy of the compounds. Suitable assays for measuring CSF stability and brain uptake are described in Examples 7 and 8, respectively.

A modulator compound of the invention can be formulated into a pharmaceutical composition wherein the modulator is the only active compound or, alternatively, the pharmaceutical composition can contain additional active compounds. For example, two or more modulator compounds may be used in combination. Moreover, a modulator compound of the invention can be combined with one or more other agents that have anti-amyloidogenic properties. For example, a modulator compound can be combined with the non-specific cholinesterase inhibitor tacrine (COGNEX®, Parke-Davis).

In another embodiment, a pharmaceutical composition of the invention is provided as a packaged formulation. The packaged formulation may include a pharmaceutical composition of the invention in a container and printed instructions for administration of the composition for treating a subject having a disorder associated with β–amyloidosis, *e.g.* Alzheimer's disease.

VI. Methods of Using Aβ Modulators

Another aspect of the invention pertains to methods for altering the aggregation or inhibiting the neurotoxicity of natural β-amyloid peptides. In the methods of the invention, natural β amyloid peptides are contacted with a β amyloid modulator such that the aggregation of the natural β amyloid peptides is altered or the neurotoxicity of the natural β amyloid peptides is inhibited. In a preferred embodiment, the modulator inhibits aggregation of the natural β amyloid peptides. In another embodiment, the modulator promotes aggregation of the natural β amyloid peptides. Preferably, aggregation of a molar excess amount of β-AP, relative to the amount of modulator, is altered upon contact with the modulator.

In the method of the invention, natural β amyloid peptides can be contacted with a modulator either *in vitro* or *in vivo*. Thus, the term "contacted with" is intended to encompass both incubation of a modulator with a natural β-AP preparation *in vitro* and delivery of the modulator to a site *in vivo* where natural β-AP is present. Since the modulator compound interacts with natural β-AP, the modulator compounds can be used to detect natural β-AP, either *in vitro* or *in vivo*. Accordingly, one use of the modulator compounds of the invention is as diagnostic agents to detect the presence of natural β-AP, either in a biological sample or *in vivo* in a subject. Furthermore, detection of natural β-AP utilizing a modulator compound of the invention further can be used to diagnose amyloidosis in a subject. Additionally, since the modulator compounds of the invention disrupt β-AP aggregation and inhibit β-AP neurotoxicity, the modulator compounds also are useful in the treatment of disorders associated with β-amyloidosis, either prophylactically or therapeutically. Accordingly, another use of the modulator compounds of the invention is as therapeutic agents to alter aggregation and/or neurotoxicity of natural β-AP.

In one embodiment, a modulator compound of the invention is used *in vitro*, for example to detect and quantitate natural β-AP in sample (*e.g.*, a sample of biological fluid). To aid in detection, the modulator compound can be modified with a detectable substance. The source of natural β-AP used in the method can be, for example, a sample of cerebrospinal fluid (*e.g.*, from an AD patient, an adult susceptible to AD due to family history, or a normal adult). The natural β-AP sample is contacted with a modulator of the invention and aggregation of the β-AP is measured, such as by the assays described in Example 2. The degree of aggregation of the β-AP sample can then be compared to that of a control sample(s) of a known concentration of β-AP, similarly contacted with the modulator and the results can be used as an indication of whether a subject is susceptible to or has a disorder associated with β-amyloidosis. Moreover, β-AP can be detected by detecting a modulating group incorporated into the modulator. For example, modulators incorporating a biotin compound as described herein (*e.g.*, an amino-terminally biotinylated β-AP peptide) can be detected using a streptavidin or avidin probe which is labeled with a detectable substance (*e.g.*, an enzyme, such as peroxidase).

In another embodiment, a modulator compound of the invention is used *in vivo* to detect, and, if desired, quantitate, natural β-AP deposition in a subject, for example to aid in the diagnosis of β amyloidosis in the subject. To aid in detection, the modulator compound can be modified with a detectable substance, preferably ^{99m}Tc or radioactive iodine (described further above), which can be detected *in vivo* in a subject. The labeled β-amyloid modulator compound is administered to the subject and, after sufficient time to allow accumulation of the modulator at sites of amyloid deposition, the labeled modulator compound is detected by standard imaging techniques. The radioactive signal generated by the labeled compound can be directly detected (*e.g.*, whole body counting), or alternatively, the radioactive signal can be converted into an image on an autoradiograph or on a computer screen to allow for imaging of amyloid deposits in the subject. Methods for imaging amyloidosis using radiolabeled proteins are known in the art. For example, serum amyloid P component (SAP), radiolabeled with either ^{123}I or ^{99m}Tc, has been used to image systemic amyloidosis (see *e.g.*, Hawkins, P.N. and Pepys, M.B. (1995) *Eur. J. Nucl. Med.* 22:595-599). Of the various isotypes of radioactive iodine, preferably ^{123}I (half-life = 13.2 hours) is used for whole body scintigraphy, ^{124}I (half life = 4 days) is used for positron emission tomography (PET), ^{125}I (half life = 60 days) is used for metabolic turnover studies and ^{131}I (half life = 8 days) is used for whole body counting and delayed low resolution imaging studies. Analogous to studies using radiolabeled SAP, a labeled modulator compound of the invention can be delivered to a subject by an appropriate route (*e.g.*, intravenously, intraspinally, intracerebrally) in a single bolus, for example containing 100 μg of labeled compound carrying approximately 180 MBq of radioactivity.

The invention provides a method for detecting the presence or absence of natural β-amyloid peptides in a biological sample, comprising contacting a biological sample with a compound of the invention and detecting the compound bound to natural β-amyloid peptides to thereby detect the presence or absence of natural β-amyloid peptides in the biological sample. In one embodiment, the β-amyloid modulator compound and the biological sample are contacted *in vitro*. In another embodiment, the β-amyloid modulator compound is contacted with the biological sample by administering the β-amyloid modulator compound to a subject. For *in vivo* administration, preferably the compound is labeled with radioactive technetium or radioactive iodine.

The invention also provides a method for detecting natural β-amyloid peptides to facilitate diagnosis of a β-amyloidogenic disease, comprising contacting a biological sample with the compound of the invention and detecting the compound bound to natural β-amyloid peptides to facilitate diagnosis of a β-amyloidogenic disease. In one embodiment, the β-amyloid modulator compound and the biological sample are contacted *in vitro*. In another embodiment, the β-amyloid modulator compound is contacted with the biological sample by administering the β-amyloid modulator compound to a subject. For *in vivo* administration, preferably the compound is labeled with radioactive technetium or radioactive iodine. Preferably, use of the method facilitates diagnosis of Alzheimer's disease.

In another embodiment, the invention provides a method for altering natural β-AP aggregation or inhibiting β-AP neurotoxicity, which can be used prophylactically or therapeutically in the treatment or prevention of disorders associated with β amyloidosis, *e.g.*, Alzheimer's Disease. Modulator compounds of the invention can reduce the toxicity of natural β-AP aggregates to cultured neuronal cells. Moreover, the modulators also have the ability to reduce the neurotoxicity of preformed Aβ fibrils. Accordingly, the modulator compounds of the invention can be used to inhibit or prevent the formation of neurotoxic Aβ fibrils in subjects (*e.g.*, prophylactically in a subject predisposed to β-amyloid deposition) and can be used to reverse β-amyloidosis therapeutically in subjects already exhibiting β-amyloid deposition.

A modulator of the invention is contacted with natural β amyloid peptides present in a subject (*e.g.*, in the cerebrospinal fluid or cerebrum of the subject) to thereby alter the aggregation of the natural β-AP and/or inhibit the neurotoxicity of the natural β-APs. A modulator compound alone can be administered to the subject, or alternatively, the modulator compound can be administered in combination with other therapeutically active agents (*e.g.*, as discussed above in subsection IV). When combination therapy is employed, the therapeutic agents can be coadministered in a single pharmaceutical composition, coadministered in separate pharmaceutical compositions or administered sequentially.

The modulator may be administered to a subject by any suitable route effective for inhibiting natural β-AP aggregation in the subject, although in a particularly preferred embodiment, the modulator is administered parenterally, most preferably to the central nervous system of the subject. Possible routes of CNS administration include intraspinal administration and intracerebral administration (*e.g.*, intracerebrovascular administration). Alternatively, the compound can be administered, for example, orally, intraperitoneally, intravenously or intramuscularly. For non-CNS administration routes, the compound can be administered in a formulation which allows for transport across the BBB. Certain modulators may be transported across the BBB without any additional further modification whereas others may need further modification as described above in subsection IV.

Suitable modes and devices for delivery of therapeutic compounds to the CNS of a subject are known in the art, including cerebrovascular reservoirs (*e.g.*, Ommaya or Rikker reservoirs; see *e.g.*, Raney, J.P. *et al.* (1988) *J. Neurosci. Nurs.* 20:23-29; Sundaresan, N. *et al.* (1989) *Oncology* 3:15-22), catheters for intrathecal delivery (*e.g.*, Port-a-Cath, Y-catheters and the like; see *e.g.*, Plummer, J.L. (1991) *Pain* 44:215-220; Yaksh, T.L. *et al.* (1986) *Pharmacol. Biochem. Behav.* 25:483-485), injectable intrathecal reservoirs (*e.g.*, Spinalgesic; see *e.g.*, Brazenor, G.A. (1987) *Neurosurgery* 21:484-491), implantable infusion pump systems (*e.g.*, Infusaid; see *e.g.*, Zierski, J. *et al.* (1988) *Acta Neurochem. Suppl.* 43:94-99; Kanoff, R.B. (1994) *J. Am. Osteopath. Assoc.* 94:487-493) and osmotic pumps (sold by Alza Corporation). A particularly preferred mode of administration is via an implantable, externally programmable infusion pump. Suitable infusion pump systems and reservoir systems are also described in U.S. Patent No. 5, 368,562 by Blomquist and U.S. Patent No. 4,731,058 by Doan, developed by Pharmacia Deltec Inc.

The method of the invention for altering β-AP aggregation *in vivo* , and in particular for inhibiting β-AP aggregation, can be used therapeutically in diseases associated with abnormal β amyloid aggregation and deposition to thereby slow the rate of β amyloid deposition and/or lessen the degree of β amyloid deposition, thereby ameliorating the course of the disease. In a preferred embodiment, the method is used to treat Alzheimer's disease (*e.g.*, sporadic or familial AD, including both individuals exhibiting symptoms of AD and individuals susceptible to familial AD). The method can also be used prophylactically or therapeutically to treat other clinical occurrences of β amyloid deposition, such as in Down's syndrome individuals and in patients with hereditary cerebral hemorrhage with amyloidosis-Dutch-type (HCHWA-D). While inhibition of β-AP aggregation is a preferred therapeutic method, modulators that promote β-AP aggregation may also be useful therapeutically by allowing for the sequestration of β-AP at sites that do not lead to neurological impairment.

Additionally, abnormal accumulation of β-amyloid precursor protein in muscle fibers has been implicated in the pathology of sporadic inclusion body myositis (IBM) (Askana, V. et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:1314-1319; Askanas, V. et al. (1995) *Current Opinion in Rheumatology* 7:486-496). Accordingly, the modulators of the invention can be used prophylactically or therapeutically in the treatment of disorders in which β-AP, or APP, is abnormally deposited at non-neurological locations, such as treatment of IBM by delivery of the modulators to muscle fibers.

This invention is further illustrated by the following examples which should not be construed as limiting. A modulator's ability to alter the aggregation of natural β-amyloid peptide and/or inhibit the neurotoxicity of natural β-amyloid peptide in the assays described below are predictive of the modulator's ability to perform the same function *in vivo*.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures, are hereby incorporated by reference.

EXAMPLE 1: Preparation of β-amyloid Modulator Compounds Comprising D-Amino Acids

β-amyloid modulators comprising D-amino acids can be prepared by solid-phase peptide synthesis, for example using an N^{α}-9-fluorenylmethyloxycarbonyl (FMOC)-based protection strategy as follows. Starting with 2.5 mmoles of FMOC-D-Val-Wang resin, sequential additions of each amino acid are performed using a four-fold excess of protected amino acids, 1-hydroxybenzotriazole (HOBt) and diisopropyl carbodiimide (DIC). Recouplings are performed when necessary as determined by ninhydrin testing of the resin after coupling. Each synthesis cycle is minimally described by a three minute deprotection (25 % piperidine/N-methyl-pyrrolidone (NMP)), a 15 minute deprotection, five one minute NMP washes, a 60 minute coupling cycle, five NMP washes and a ninhydrin test. For N-terminal modification, an N-terminal modifying reagent is substituted for an FMOC-D-amino acid and coupled to a 700 mg portion of the fully assembled peptide-resin by the above protocol. The peptide is removed from the resin by treatment with trifluoroacetic acid (TFA) (82.5 %), water (5 %), thioanisole (5 %), phenol (5 %), ethanedithiol (2.5 %) for two hours followed by precipitation of the peptide in cold ether. The solid is pelleted by centrifugation (2400 rpm x 10 min.), and the ether decanted. The solid is resuspended in ether, pelleted and decanted a second time. The solid is dissolved in 10 % acetic acid and lyophilized to dryness. For preparative purification and subsequent analytical characterization, 60 mg of the solid is dissolved in 25 % acetonitrile (ACN) /0.1 % TFA and applied to a C18 reversed phase high performance liquid chromatography (HPLC) column.

Alternatively, *β*-amyloid modulators comprising D-amino acids can be prepared on a Rainin PS3 peptide synthesizer using an automated protocol established by the manufacturer for a 0.25 mmole scale synthesis. Couplings are performed using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium-hexafluoro-phosphate (HBTU) / FMOC-D-amino acid in four fold excess in 0.4 M N-methylmorpholine (NMM) / dimethylformamide (DMF) for 60 minutes. In between couplings, the FMOC group is removed by reaction with 20% piperidine / DMF for 20 minutes. The peptide is removed from the resin by treatment with 95% TFA/water for one hour and precipitated with ether. The pellet is resuspended in 40% acetonitrile/water and lyophilized. When necessary, the material was purified by preparative HPLC using 15%-50% acetonitrile over 60 minutes on a Vydac C18 column (21 x 250 mm).

Various *N*-terminally modified β-amyloid modulator compounds can be synthesized using standard methods. Fully-protected resin-bound peptides are prepared as described above on an appropriate resin to eventually afford carboxyl terminal peptide acids. Small portions of each peptide resin (*e.g.*, 13-20 μmoles) are aliquoted into separate reaction vessels. The *N*-terminal FMOC protecting group of each sample is removed in the standard manner with 20 % piperidine in NMM followed by extensive washing with DMF. The unprotected *N*-terminal α-amino group of each peptide-resin sample can be modified using one of the following methods:

Method A, coupling of modifying reagents containing free carboxylic acid groups: The modifying reagent (five equivalents) is predissolved in NMP, DMSO or a mixture of these two solvents. HOBT and DIC (five equivalents of each reagent) are added to the dissolved modifier and the resulting solution is added to one equivalent of free-amino peptide-resin. Coupling is allowed to proceed overnight, followed by washing. If a ninhydrin test on a small sample of peptide-resin shows that coupling is not complete, the coupling is repeated using 1-hydroxy-7-azabenzotriazole (HOAt) in place of HOBt.

Method B, coupling of modifying reagents obtained in preactivated forms: The modifying reagent (five equivalents) is predissolved in NMP, DMSO or a mixture of these two solvents and added to one equivalent of peptide-resin. Diisopropylethylamine (DIEA; six equivalents) is added to the suspension of activated modifier and peptide-resin. Coupling is allowed to proceed overnight, followed by washing. If a ninhydrin test on a small sample of peptide-resin shows that coupling is not complete, the coupling is repeated.

After the second coupling (if required) the *N*-terminally modified peptide-resins are dried at reduced pressure and cleaved from the resin with removal of side-chain protecting groups as described above. Analytical reversed-phase HPLC is used to confirm that a major product is present in the resulting crude peptides, which are purified using Millipore Sep-Pak cartridges or preparative reverse-phase HPLC. Mass spectrometry or high-field nuclear magnetic resonance spectrometry is used to confirm the presence of the desired compound in the product.

Method C, preparation of N-terminal-alkyl substituted peptides using bromoacetyl peptide intermediates: A resin-bound peptide can be coupled to bromoacetic acid (12 equivalents) with 1,3-diisopropylcarbodiimide (DIC) (13 equivalents) in DMF. The resulting bromoacetyl substituted peptide can be modified upon reaction with primary or secondary amines including, methylamine, ethylamine, propylamine, isopropylamine and piperidine. The reaction is performed in 60% DMSO/DMF and is typically complete after 24 hours.

Method D, preparation of N-terminal-alkyl substituted peptides via reductive alkylation: After the peptide is dissolved (or partially dissolved) in water containing 0-10 % methanol, it is reacted with an aldehyde (5-8 equivalents) and sodiumcyanoborohydride (10-16 equivalents). The number of equivalents can be adjusted for the type of aldehyde and the degree of substitution desired. The pH of the resulting solution is adjusted to 2 with 1 M HCl and maintained at 2 for one hour. The reaction is monitored by hplc and is usually completed with two hours. The reaction mix is concentrated at room temperature and hplc purified.

Method E, C-terminal modification: The peptide was synthesized on 2-chlorotrityl resin using standard Fmoc chemistry however the final D-amino acid group coupled was Boc protected. The peptide was removed from the resin with 8/1/1 dichloromethane (DCM) / acetic acid / trifluoroethanol and the mixture concentrated. The peptide residue was dissolved in 20 % acetonitrile, frozen and lyophilyzed overnight. The crude BOC protected peptide acid was coupled under basic conditions (pH=11, adjusted with DIEA) to an amine with one equivalent each of 1-hydroxy-7-

azobenzotriazole(HOAt) and DIC. The reaction was completed after stirring overnight and the peptide precipitated with water. The BOC group was cleaved upon reaction with 25 % TFA in DCM for one hour and the peptide HPLC purified.

EXAMPLE 2: β-Amyloid Aggregation Assays

The ability of β-amyloid modulator compounds to modulate (*e.g.*, inhibit or promote) the aggregation of natural β-AP when combined with the natural β-AP can be examined in one or both of the aggregation assays described below. Natural β-AP (β-AP_{1-40}) for use in the aggregation assays is commercially available from Bachem (Torrance, CA).

A. Nucleation Assay

The nucleation assay is employed to determine the ability of test compounds to alter (*e.g.* inhibit) the early events in formation of β-AP fibers from monomeric β-AP. Characteristic of a nucleated polymerization mechanism, a lag time is observed prior to nucleation, after which the peptide rapidly forms fibers as reflected in a linear rise in turbidity. The time delay before polymerization of β-AP monomer can be quantified as well as the extent of formation of insoluble fiber by light scattering (turbidity). Polymerization reaches equilibrium when the maximum turbidity reaches a plateau. The turbidity of a solution of natural β-AP in the absence or presence of various concentrations of a β-amyloid modulator compound is determined by measuring the apparent absorbance of the solution at 405nm ($A_{405\ nm}$) over time. The threshold of sensitivity for the measurement of turbidity is in the range of 15-20 μM β-AP. A decrease in turbidity over time in the presence of the modulator, as compared to the turbidity in the absence of the modulator, indicates that the modulator inhibits formation of β-AP fibers from monomeric β-AP. This assay can be performed using stirring or shaking to accelerate polymerization, thereby increasing the speed of the assay. Moreover the assay can be adapted to a 96-well plate format to screen multiple compounds.

To perform the nucleation assay, first A$\beta_{1\text{-}40}$ peptide is dissolved in HFIP (1,1,1,3,3,3-Hexafluoro-2-propanol; Aldrich 10,522-8) at a concentration of 2 mg peptide/ml and incubated at room temperature for 30 min. HFIP-solubilized peptide is sonicated in a waterbath sonicator for 5 min at highest setting, then evaporated to dryness under a stream of argon. The peptide film is resuspended in anhydrous dimethylsulfoxide (DMSO) at a concentration of 6.9 mg/ml (25x concentration), sonicated for 5 min as before, then filtered through a 0.2 micron nylon syringe filter (VWR cat. No. 28196-050). Test compounds are dissolved in DMSO at a 100x concentration. Four volumes of 25x A$\beta_{1\text{-}40}$ peptide in DMSO are combined with one volume of test compound in DMSO in a glass vial, and mixed to produce a 1:1 molar ratio of Aβ peptide to test compound. For different molar ratios, test compounds are diluted with DMSO prior to addition to A$\beta_{1\text{-}40}$, in order to keep the final DMSO and A$\beta_{1\text{-}40}$ concentrations constant. Control samples do not contain the test compound. Ten microliters of the mixture is then added to the bottom of a well of a Corning Costar ultra low binding 96-well plate (Corning Costar, Cambridge MA; cat. No. 2500). Ninety microliters of water is added to the well, the plate is shaken on a rotary shaken at a constant speed at room temperature for 30 seconds, an additional 100 μl of 2x PTL buffer (20 mM NaH_2PO_4, 300 mM NaCl, pH 7.4) is added to the well, the plate is reshaken for 30 seconds and a baseline (t=0) turbidity reading is taken by measuring the apparent absorbance at 405 nm using a Bio-Rad Model 450 Microplate Reader. The plate is then returned to the shaker and shaken continuously for 5 hours. Turbidity readings are taken at 15 minute intervals.

β-amyloid aggregation in the absence of any modulators results in enhanced turbidity of the natural β-AP solution (*i.e.*, an increase in the apparent absorbance at 405 nm over time). Accordingly, a solution including an effective inhibitory modulator compound exhibits reduced turbidity as compared to the control sample without the modulator compound (*i.e.*, less apparent absorbance at 405 nm over time as compared to the control sample).

Alternative to use of turbidity to quantitate β-amyloid aggregation, fluorescence of thioflavin T (Th-T) also can be used to quantitate β-amyloid aggregation in the nucleation assay (use of Th-T fluorescence for quantitating β-amyloid aggregation is described further below for the seeded extension assay).

B. Fibril Binding Assay

The following materials are needed for the Fibril binding assay: Millipore multifilter apparatus; 12 x 75 glass tubes; GF/F 25 mm glass filters; PBS/0.1% tween 20 at 4°C (PBST); Aβ fibrils; radioactive compound; nonradioactive compound; Eppendorf repeat pipettor with tips; labels; forceps; and vacuum.

In this assay, each sample is run in triplicate. The “aged” Aβ fibril is first prepared approximately 8 days in advance by aging 1 ml aliquots of a 200 μM Aβ 1-40 peptide solution in 4%DMSO/PBS for 8 days at 37°C with rocking. Such "aged" Aβ peptide can be tested directly on cells or frozen at -80°C.

The 200 μM Aβ fibril is diluted in PBST to yield a 4 μM solution (320 μl in 16 ml PBST). 100μL aliquots of this solution are added per tube with the repeat pipettor.

The β-amyloid modulator compounds of the invention are prepared at 2μM – 200 fM dilutions as follows:

Dilute a 5 mM stock 1:3 in DMSO to yield a 1.6667 stock (200 μl in 400 μl DMSO).

Dilute a 1.667 mM stock 1:3 in DMSO to yield a 0.5556 stock (200 μl in 400 μl DMSO).

Dilute a 555.556 μM stock 1:3 in DMSO to yield a 185.19 stock (200 μl in 400 μl DMSO).

5 Dilute a 185.185 μM stock 1:3 in DMSO to yield a 61.728 stock (200 μl in 400 μl DMSO).

Dilute a 61.728 μM stock 1:3 in DMSO to yield a 20.576 stock (200 μl in 400 μl DMSO).

Dilute a 20.576 μM stock 1:3 in DMSO to yield a 6.8587 stock (200 μl in 400 μl DMSO).

Dilute a 6.859 μM stock 1:3 in DMSO to yield a 2.2862 stock (200 μl in 400 μl DMSO).

Dilute a 2.286 μM stock 1:3 in DMSO to yield a 0.7621 stock (200 μl in 400 μl DMSO).

10 Dilute a 762.079 nM stock 1:3 in DMSO to yield a 254.03 stock (200 μl in 400 μl DMSO).

Dilute a 254.026 nM stock 1:3 in DMSO to yield a 84.675 stock (200 μl in 400 μl DMSO).

Dilute a 84.675 nM stock 1:3 in DMSO to yield a 28.225 stock (200 μl in 400 μl DMSO).

Dilute a 28.225 nM stock 1:3 in DMSO to yield a 9.4084 stock (200 μl in 400 μl DMSO).

Dilute a 9.408 nM stock 1:3 in DMSO to yield a 3.1361 stock (200 μl in 400 μl DMSO).

15 Dilute a 3.136 nM stock 1:3 in DMSO to yield a 1.0454 stock (200 μl in 400 μl DMSO).

Dilute a 1.045 nM stock 1:3 in DMSO to yield a 0.3485 stock (200 μl in 400 μl DMSO).

Dilute a 348.459 pM stock 1:3 in DMSO to yield a 116.15 stock (200 μl in 400 μl DMSO).

Dilute a 116.153 pM stock 1:3 in DMSO to yield a 38.718 stock (200 μl in 400 μl DMSO).

Dilute 185.185 μM stock 1:25 in PBST to yield 7.4074 (50 μL in 1.2 mL PBST)

20 Dilute 61.728 μM stock 1:25 in PBST to yield 2.4691 (50 μL in 1.2 mL PBST)

Dilute 20.576 μM stock 1:25 in PBST to yield 0.823 (50 μL in 1.2 mL PBST)

Dilute 6.859 μM stock 1:25 in PBST to yield 0.2743 (50 μL in 1.2 mL PBST)

Dilute 2.286 μM stock 1:25 in PBST to yield 0.0914 (50 μL in 1.2 mL PBST)

Dilute 762.079 nM stock 1:25 in PBST to yield 30.483 (50 μL in 1.2 mL PBST)

25 Dilute 254.026 nM stock 1:25 in PBST to yield 10.161 (50 μL in 1.2 mL PBST)

Dilute 84.675 nM stock 1:25 in PBST to yield 3.387 (50 μL in 1.2 mL PBST)

Dilute 28.225 nM stock 1:25 in PBST to yield 1.129 (50 μL in 1.2 mL PBST)

Dilute 9.408 nM stock 1:25 in PBST to yield 0.3763 (50 μL in 1.2 mL PBST)

Dilute 3.136 nM stock 1:25 in PBST to yield 0.1254 (50 μL in 1.2 mL PBST)

30 Dilute 1.045 nM stock 1:25 in PBST to yield 0.0418 (50 μL in 1.2 mL PBST)
Dilute 348.459 pM stock 1:25 in PBST to yield 13.938 (50 μL in 1.2 mL PBST)
Dilute 116.153 pM stock 1:25 in PBST to yield 4.6461 (50 μL in 1.2 mL PBST)

The β-amyloid modulator compound (200μL) is then added to the appropriate tube containing the Aβ fibril.

The radioactively labeled β-amyloid modulator compound is prepared using standard radioactive safety protocols by making a dilution into a PBS/0.1% tween-20 solution such that there is a final concentration of 20,000 dpm per 100 μL. 100 μl aliqouots of the radioactively labeled β-amyloid modulator compound are added per tube using the repeat pipettor. The samples are covered with parafilm and incubated at 37 °C inside plastic radioactivity bags overnight.

To filter the samples, the filters are pre-wetted in a small volume of PBST. Two Millipore multifiltration apparati are set with GF/F filters in each filtration slot following the instructions from the manufacturer. The samples are removed from the 37 °C incubator and each sample is filtered using a small volume (~5 ml) of cold PBST buffer. The sample tube is then washed with two additional 5 mL volumes of cold PBST buffer. The vacuum is allowed to pull to a semi dry filter for approximately 2 minutes after adding the last sample and the filter is transferred to a labelled tube for iodination counting. One minute counts are recorded, the data is plotted, and the Prism program (GraphPAD) is used to analyze the graph, according to the manufacturer's instrutions.

C. Seeded Extension Assay

The seeded extension assay can be employed to measure the rate of Aβ fiber formed in a solution of Aβ monomer following addition of polymeric Aβ fiber "seed". The ability of test compounds to prevent further deposition of monomeric Aβ to previously deposited amyloid is determined using a direct indicator of β-sheet formation using fluorescence. In contrast with the nucleation assay, the addition of seed provides immediate nucleation and continued growth of preformed fibrils without the need for continuous mixing, and thus results in the absence of a lag time before polymerization starts. Since this assay uses static polymerization conditions, the activity of positive compounds in the nucleation assay can be confirmed in this second assay under different conditions and with an additional probe of amyloid structure.

In the seeded extension assay, monomeric Aβ_{1-40} is incubated in the presence of a "seed" nucleus (approximately ten mole percent of Aβ that has been previously allowed to polymerize under controlled static conditions). Samples of the solution are then diluted in thioflavin T (Th-T). The polymer-specific association of Th-T with Aβ produces a fluorescent complex that allows the measurement of the extent of fibril formation (Levine, H. (1993) *Protein Science* 2:404-410). In particular, association of Th-T with aggregated β-AP, but not monomeric or loosely associated β-AP, gives rise to a new excitation (ex) maximum at 450 nm and an enhanced emission (em) at 482 nm, compared to the 385 nm (ex) and 445 nm (em) for the free dye. Small aliquots of the polymerization mixture contain sufficient fibril to be mixed with Th-T to allow the monitoring of the reaction mixture by repeated sampling. A linear growth curve is observed in the presence of excess monomer. The formation of thioflavin T responsive β-sheet fibrils parallels the increase in turbidity observed using the nucleation assay.

A solution of Aβ monomer for use in the seeded extension assay is prepared by dissolving an appropriate quantity of Aβ_{1-40} peptide in 1/25 volume of dimethysulfoxide (DMSO), followed by water to 1/2 volume and 1/2 volume 2x PBS (10x PBS: NaCl 137 mM, KCl 2.7 mM $Na_2HPO_4 \cdot 7H_2O$ 4.3 mM, KH_2PO_4 1.4 mM pH 7.2) to a final concentration of 200 μM. To prepare the stock seed, 1 ml of the Aβ monomer preparation, is incubated for approximately 8 days at 37 °C and sheared sequentially through an 18, 23, 26 and 30 gauge needle 25, 25, 50, and 100 times respectively. 2 μl samples of the sheared material is taken for fluorescence measurements after every 50 passes through the 30 gauge needle until the fluorescence units (FU) plateau (approx. 100-150x). Test compounds are prepared by dissolving an appropriate amount of test compound in 1x PBS to a final concentration of 1 mM (10x stock). If insoluble, the compound is dissolved in 1/10 volume of DMSO and diluted in 1x PBS to 1 mM. A further 1/10 dilution is also prepared to test each candidate at both 100 μM and 10 μM.

To perform the seeded extension assay, each sample is set up with 50 µl of 200 µM monomer, 125 FU sheared seed (a variable quantity dependent on the batch of seed, routinely 3-6 µl) and 10 µl of 10x modulator solution. The sample volume is then adjusted to a final volume of 100 µl with 1x PBS. Two concentrations of each modulator typically are tested: 100 µM and 10 µM, equivalent to a 1:1 and a 1:10 molar ratio of monomer to modulator. The controls include an unseeded reaction to confirm that the fresh monomer contains no seed, and a seeded reaction in the absence of any modulators, as a reference to compare against candidate modulators. The assay is incubated at 37 °C for 6 h, taking 2 µl samples hourly for fluorescence measurements. To measure fluorescence, a 2 µl sample of Aβ is added to 400 µl of Thioflavin-T solution (50 mM Potassium Phosphate 10 mM Thioflavin-T pH 7.5). The samples are vortexed and the fluorescence is read in a 0.5 ml micro quartz cuvette at EX 450 nm and EM 482 nm (Hitachi 4500 Fluorimeter).

β-amyloid aggregation results in enhanced emission of Thioflavin-T. Accordingly, samples including an effective inhibitory modulator compound exhibit reduced emission as compared to control samples without the modulator compound.

EXAMPLE 3: Analysis of β-Amyloid Modulator Compounds

In this example, β-amyloid modulator compounds described herein were prepared and tested for their ability to inhibit aggregation of natural β-amyloid peptide using aggregations assays as described in Example 2. The results from a first series of experiments, are summarized below in Tables I, II, and III.

TABLE I

PPI#	Nucleation assay Δ lag			Fibril binding Kd's		
	5 μM	2.5 μM	1.25 μM	cmpd	ref cmpd	ref Kd
803	<1	<1	<1			
913	1	1	1			
968	>5	>5	2			
969	>5	>5	3	1.13×10^{-9}	PPI-558	3.7×10^{-9}
970	>5	>5	1			
992	3	1	1	2.43×10^{-9}	PPI-558	3.70×10^{-9}
993	1	1	1			
1005	3	3	1			
1006	1	1	1			
*1007	4	4	3	8.64×10^{-10}	PPI-558	1.69×10^{-9}
#1007	1.5	1.5	1.5	6.27×10^{-10}	PPI-558	2.75×10^{-9}
1008				1.75×10^{-9}	PPI-558	1.00×10^{-9}
#1013	2	>3	2	2.47×10^{-10}	PPI-558	1.69×10^{-9}
1017				3.89×10^{-10}	PPI-558	2.42×10^{-9}
1018				7.01×10^{-10}	PPI-558	2.42×10^{-9}
1020				6.01×10^{-10}	PPI-558	2.42×10^{-9}
1022				1.50×10^{-10}	PPI-558	1.00×10^{-9}
1025				4.30×10^{-10}	PPI-558	1.00×10^{-9}
1028				4.90×10^{-10}	PPI-558	1.00×10^{-9}
1038				6.52×10^{-10}	PPI-558	3.76×10^{-9}
1039				2.44×10^{-9}	PPI-558	3.76×10^{-9}
1040				4.08×10^{-10}	PPI-558	2.4×10^{-9}
1041				1.61×10^{-9}	PPI-558	2.4×10^{-9}
1042				2.34×10^{-10}	PPI-558	2.4×10^{-9}
1088				3.40×10^{-9}	PPI-558	1.93×10^{-9}

1089				5.7×10^{-10}	PPI-558	3.3×10^{-9}
1093				1.02×10^{-9}	PPI-558	1.93×10^{-9}
1094				3.7×10^{-9}	PPI-558	3.5×10^{-9}
1179				6.04×10^{-10}	PPI-558	1.93×10^{-9}
1180				3.3×10^{-10}	PPI-558	3.5×10^{-9}
1261				1.12×10^{-8}	PPI-558	3.34×10^{-9}

Notes:

* means nucleation assay data was measured at 3, 1 and 0.3 µM of compound

means nucleation assay data was measured at 2.5, 1.25 and 0.6 µM of compound

TABLE II

PPI#	Nucleation assay data			Fibril binding Kd's		
	3 µM	1 µM	0.3 µM	cmpd	ref cmpd	ref Kd
*1019	>2.5	>2.5	2.0	4.11×10^{-10}	PPI-558	1.69×10^{-9}
1019				5.34×10^{-10}	PPI-558	1.93×10^{-9}
1301				1.1×10^{-9}	PPI-1318	1.4×10^{-9}
1302				2.2×10^{-10}	PPI-1318	1.4×10^{-9}
1303				1.1×10^{-9}	PPI-1318	1.4×10^{-9}
1318	>5	2	1	7.7×10^{-11}	PPI-558	2.3×10^{-9}
1318				1.4×10^{-9}		
1318				6.2×10^{-11}		
1319	>5	>5	1			
1320	>5	3	1	1.4×10^{-9}	PPI-1318	6.2×10^{-11}
1321	<1	<1	<1			
1322				1.2×10^{-9}	PPI-1318	6.2×10^{-11}
1323						
1324						
1325				1.4×10^{-9}	PPI-1318	1.4×10^{-9}

1326				5.6 x 10^{-10}	PPI-1318	6.2 x 10^{-11}
1327				8.2 x 10^{-10}	PPI-1318	1.4 x 10^{-9}
1328				2.4 x 10^{-9}	PPI-1318	6.2 x 10^{-11}
1329						
*1125	>2.5	>2.5	2.0	1.27 x 10^{-9}	PPI-558	2.08 x 10^{-9}
1125				1.34 x 10^{-9}	PPI-558	5.05 x 10^{-9}
1133				3.18 x 10^{-7}	PPI-558	2.08 x 10^{-9}
1155				1.24 x 10^{-7}	PPI-558	2.08 x 10^{-9}

Notes:

* means nucleation assay data was measured at 2.5, 1.25 and 0.6 μM of compound

The modulator compounds were evaluated in nucleation assays utilizing 5 μM $A\beta_{1-40}$ and either 5 μM, 2.5 μM, 1.25 μM, 3 μM, 1 μM, or 0.3 μM test compound. The change in lag time (ΔLag) is presented as the ratio of the lag time observed in the presence of the test compound (at either 5 μM, 2.5 μM, 1.25 μM, 3 μM, 1 μM, or 0.3 μM) to the lag time of the control.

TABLE III

PPI #	STRUCTURE	Fibril binding Kd's cmpd
PPI-504	TFA • H-(lv-[3-I]y-fa)-NH_2	
PPI-1181	TFA • H-(lvffl)-NH-Et	
PPI-1465	TFA • H-lvffl-NH-CH_2CH_2-NH_2	3.6 x 10^{-9}
PPI-1603	TFA• H-(GGClvffl)-NH_2	
PPI-1604	TFA• H-(GGClvfyl)-NH_2	
PPI-1605	TFA• H-(GGClvf-[3-I]y-l)-NH_2	
PPI-1619	2TFA • H-LVF-NH-NH-FVL-H	3.5 x 10^{-8} (an analog of 1125)

PPI-1621	2TFA • H-LVF-NH-NH-fvl-H	8.7×10^{-9} (an analog of 1125)
PPI-1635	TFA • H-lff-(nvl)-l-NH_2	1.4×10^{-9}
PPI-1636	TFA • H-lf-[pF]f-(nvl)-l-NH_2	1.5×10^{-9}
PPI-1637	TFA • H-l-[pF]f-[pF]f-(nvl)-l-NH_2	1.8×10^{-9}
PPI-1782	TFA•Me-lvyfl-NH_2	
PPI-1783	TFA•H-(lvyfl)-NH_2	
PPI-1784	TFA• Me-(lv-[*p*-F]f-fl)-NH_2	2.5×10^{-9}
PPI-1785	TFA•H-(lv-[*p*-F]f-fl)-NH_2	2.8×10^{-9}
PPI-1786	TFA•H-(lvf-[*p*-F]f-l)-NH_2	
PPI-1787	TFA•Me-lvff-[nvl])-NH_2	5.8×10^{-9}
PPI-1788	TFA• Me-(lvff-[nle])-NH_2	($\sim 4 \times 10^{-9}$) 3-point assay
PPI-1799	TFA•Me-lvffl)-OH	
PPI-1800	TFA• Me-(lvffl)-NH-OH	($\sim 4 \times 10^{-9}$) 3-point assay
PPI-1805	TFA • H-(lv-[*p*-F]f-f-(nvl))-NH_2	
PPI-1806	TFA • Me-(l-v-[*p*-F]f-f-(nvl))-NH_2	
PPI 1807	TFA • H-((nvl)-v-[*p*-F]f-f-nvl)-NH_2	
PPI-1818	TFA • H-(l-(nvl)-[*p*-F]f-f-(nvl)-NH_2	
PPI 1819	TFA • H-((nvl)-(nvl)-[*p*-F]f-f-(nvl))-NH_2	
PPI 1820	TFA • Me-(l-(nvl)-[*p*-F]f-f-(nvl))-NH_2	
PPI 1827	TFA • H-(lvff-(nvl))-NH_2	
PPI 1828	Ac-(lvffl)-NH_2	
PPI 1829	Ac-(lvffl)-OH	
PPI 1830	TFA • H-(lv-[3-I]y-fl)-NH_2	

(nvl) = D-norvaline
(nle) = D-norleucine
[3-I]y = 3-iodo-D-tyrosine
[p-F]f = para-fluoro-D-phenylalanine

PPI-1801 is the acetyl amide analog of H-LPFFD-OH that has been reported in the literature. This compound was prepared and tested for activity for comparison purposes. The results indicate that this compound binds poorly to fibrils in the assay used herein.

In contrast, the results shown in Tables I, II, and III, and Figure 2 demonstrate that β-amyloid modulators of the invention are effective inhibitors of Aβ aggregation.

EXAMPLE 6: **Neurotoxicity Assay**

The neurotoxicity of natural β-amyloid peptide aggregates, in either the presence or absence of a β-amyloid modulator, can be tested in a cell-based assay using either a rat or human neuronally-derived cell line (PC-12 cells or NT-2 cells, respectively) and the viability indicator 3,(4,4-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT). (See *e.g.*, Shearman, M.S. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:1470-1474; Hansen, M.B. *et al.* (1989) *J. Immun. Methods* 119:203-210 for a description of similar cell-based viability assays). PC-12 is a rat adrenal pheochromocytoma cell line and is available from the American Type Culture Collection, Rockville, MD (ATCC CRL 1721). MTT (commercially available from Sigma Chemical Co.) is a chromogenic substrate that is converted from yellow to blue in viable cells, which can be detected spectrophotometrically.

To test the neurotoxicity of natural β-amyloid peptides, stock solutions of fresh Aβ monomers and aged Aβ aggregates are first prepared. $A\beta_{1-40}$ in 100% DMSO is prepared from lyophilized powder and immediately diluted in one half the final volume in H_20 and then one half the final volume in 2X PBS so that a final concentration of 200 μM peptide, 4% DMSO is achieved. Peptide prepared in this way and tested immediately on cells is referred to as "fresh" Aβ monomer. To prepare "aged" Aβ aggregates, peptide solution is placed in a 1.5 ml Eppendorf tube and incubated at 37 °C for eight days to allow fibrils to form. Such "aged" Aβ peptide can be tested directly on cells or frozen at -80°C. The neurotoxicity of fresh monomers and aged aggregates are tested using PC12 and NT2 cells. PC12 cells are routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum, 5% fetal calf serum, 4mM glutamine, and 1% gentamycin. NT2 cells are routinely cultured in OPTI-MEM medium (GIBCO BRL CAT. #31985) supplemented with 10% fetal calf serum, 2 mM glutamine and 1% gentamycin. Cells are plated at 10-15,000 cells per well in 90 μl of fresh medium in a 96 -well tissue culture plate 3-4 hours prior to treatment. The fresh or aged Aβ peptide solutions (10 μL) are then diluted 1:10 directly into tissue culture medium so that the final concentration is in the range of 1-10 μM peptide. Cells are incubated in the presence of peptide without a change in media for 48 hours at 37°C. For the final three hours of exposure of the cells to the β-AP preparation, MTT is added to the media to a final concentration of 1 mg/ml and incubation is continued at 37 °C. Following the two hour incubation with MTT, the media is removed and the cells are lysed in 100 μL isopropanol/0.4N HCl with agitation. An equal volume of PBS is added to each well and the plates are agitated for an additional 10 minutes. Absorbance of each well at 570 nm is measured using a microtiter plate reader to quantitate viable cell.

Using this assay, the neurotoxicity of aged (5 day or 8 day) $A\beta_{1-40}$ aggregates alone, but not fresh $A\beta_{1-40}$ monomers alone, was confirmed. Experiments demonstrated that incubating the neuronal cells with increasing amounts of fresh $A\beta_{1-40}$ monomers was not significantly toxic to the cells whereas incubating the cells with increasing amounts of 5 day or 8 day $A\beta_{1-40}$ aggregates led to increasing amount of neurotoxicity. The EC_{50} for toxicity of aged $A\beta_{1-40}$ aggregates was 1-2 μM for both the PC12 cells and the NT2 cells.

To determine the effect of a β-amyloid modulator compound on the neurotoxicity of $A\beta_{1-40}$ aggregates, a modulator compound is preincubated with $A\beta_{1-40}$ monomers under standard nucleation assay conditions as described in Example 2 and at particular time intervals post-incubation, aliquots of the β-AP/modulator solution are removed and 1) the turbidity of the solution is assessed as a measure of aggregation and 2) the solution is applied to cultured neuronal cells for 48 hours at which time cell viability is assessed using MTT to determine the neurotoxicity of the solution. Additionally, the ability of β-amyloid modulator compounds to reduce the neurotoxicity of preformed $A\beta_{1-40}$ aggregates can be assayed. In these experiments, $A\beta_{1-40}$ aggregates are preformed by incubation of the monomers in the absence of any modulators. The modulator compound is then incubated with the preformed $A\beta_{1-40}$ aggregates for 24 hours at 37 °C, after which time the β-AP/modulator solution is collected and its neurotoxicity evaluated as described above.

EXAMPLE 7: Assay of Modulator Compound Stability in Cerebrospinal Fluid

The stability of a modulator compound in cerebrospinal fluid (CSF) can be assayed in an *in vitro* assay as follows. A CSF solution is prepared containing 75% Rhesus monkey CSF (commercially available from Northern Biomedical Research), 23% sterile phosphate buffered saline and 2% dimethylsulfoxide (v/v) (Aldrich Chemical Co., Catalog No. 27,685-5). Test modulator compounds are added to the CSF solution to a final concentration of 40 μM or 15 μM. All sample handling is carried out in a laminar flow hood and test solutions are maintained at 37 °C during the assay. After 24 hours, enzymatic activity in the solutions is quenched by adding acetonitrile to produce a final concentration of 25% (v/v). Samples (at the 0 time point and the 24 hour time point) are analyzed at room temperature using reverse-phase HPLC. A microbore column is used to maximize sensitivity. The parameters for analytical HPLC are as follows:

Solvent System

A: 0.1% Trifluoroacetic acid (TFA) in water (v/v)
B: 0.085% TFA/Acetonitrile, 1% H_2O (v/v)

Injection and Gradient

Inject: 100-250 μL of test sample

Run: 10% for B for 5 min., then 10-70% B over 60 min.

Chromatographic analysis is performed using a Hewlett Packard 1090 series II HPLC. The column used for separation is a C4, 5 μm, 1 x 250 mm (Vydac #214TP51). The flow rate is 50 μL/min and the elution profile of the test compounds is monitored at 214, 230, 260 and 280 nm.

EXAMPLE 8: Brain Uptake Assay

Brain levels of our Aß-derived peptides were determined in the rat following intravenous administration. Under ketamine/xylazine anesthesia male Sprague-Dawley rats (219-302g) received an intravenous injection via a catheter inserted in the left jugular vein (dose volume of 4 mL/kg administered over 1 minute) The actual dose administered of each compound tested is shown in Figure 1.

At 60 minutes post administration the left common carotid artery was cannulated to enable perfusion of the left forebrain to remove cerebral blood. The left forebrain, void of blood was subjected to capillary depletion as described by (Triguero et al. (1990) *J. Neurochem.* 54:1882-1888). This established technique separates brain vasculature from the parenchyma and, thus, allows the accurate determination of the concentration of compound under investigation that has traversed the blood brain barrier. The amount of parent compound that was present within the brain was determined by LC/MS/MS.

The above-described assay was used to measure the brain uptake of the following modulators:

Compounds PPI	Structure	mwt	Conc (mg/mL)	Dose mg/kg IV
1324	TFA. H-(l-[F$_5$]f-fvl)-NH2	841	1.20	4.9
1318	TFA. H-(lf-D-Cha-vl)-NH2	757	0.29	1.0
1319	TFA. H-(lf-[p-F]f-vl)-NH2	769	1.70	6.6
1327	TFA. H-(l-[p-F]f-[p-F]f-vl)-NH2	787	0.98	4.0
1301	TFA. H-(lvf-D-Cha-l)-NH2	757	0.70	2.9
1302	TFA. H-(lvf-[p-F]f-l)-NH2	769	0.19	0.7
1328	TFA. H-(l-[F$_5$]f-[F$_5$]f-vl)-NH2	931	0.29	1.2
1322	TFA. H-(l-D-Cha-fvl)-NH2	757	0.03	0.1
1303	TFA. H-(lvf-[F$_5$]f-l)-NH2	841	0.27	1.0
1326	TFA. H-(l-D-Cha-D-Cha-vl)-NH2	763	0.05	0.2
1320	TFA. H-(lf-[F$_5$]f-vl)-NH2	841	0.70	3.0

*The lower letter notation refers to a D-configuration.

The results are summarized in Figure 1.

The β-amyloid modulator compounds described herein are summarized in the following Table.

TABLE IV

PPI#	Description	SEQ ID NO
803	TFA•N,N-dimethyl-(Gaffvl)-NH$_2$	
913	TFA•N,N-dimethyl-(affvl)-NH$_2$	
918	TFA•H-(l-[Me]v-ffa)-NH$_2$	
968	TFA•N-methyl-(Gaffvl)-NH$_2$	
969	TFA•N-ethyl-(Gaffvl)-NH$_2$	
970	TFA•N-isopropyl-(Gaffvl)-NH$_2$	
992	TFA•H-(lvffa)-isopropylamide	
993	TFA•H-(lvffa)-dimethylamide	
1005	TFA•N,N-diethyl-(Gaffvl)-NH$_2$	
1006	TFA•N,N-diethyl-(affvl)-NH$_2$	
1007	TFA•N,N-dimethyl-(lvffl)-NH$_2$	
1008	TFA•N,N-dimethyl-(lffvl)-NH$_2$	
1013	TFA•H-(Glvffl)-NH$_2$	
1017	TFA•N-ethyl-(Glvffl)-NH$_2$	
1018	TFA•N-ethyl-(Glffvl)-NH$_2$	
1020	TFA•N-methyl-(lffvl)-NH$_2$	
1022	TFA•N-ethyl-(lvffl)-NH$_2$	
1025	TFA•N-propyl-(lvffl)-NH$_2$	
1028	TFA•N,N-diethyl-(Glvffl)-NH$_2$	
1038	TFA•H-(ivffi)-NH$_2$	
1039	TFA•H-(ivffa)-NH$_2$	
1040	TFA•H-(iiffi)-NH$_2$	
1041	TFA•H-(D-Nle-vffa)-NH$_2$	
1042	TFA•H-(D-Nle-vff- D-Nle)-NH$_2$	
1088	TFA•1-piperidine-acetyl-(lvffl)-NH$_2$	
1089	TFA•1-piperidine-acetyl-(lffvl)-NH$_2$	
1093	TFA•H-lvffl-isopropylamide	

1094	TFA•H-lffvl-isopropylamide	
1179	TFA•H-(lvffl)-methylamide	
1180	TFA•H-(lffvl)-methylamide	
1261	TFA•H-(lvffl)-OH	
1019	TFA•N-methyl-(lvffl)-NH_2	
1301	TFA•H-(lvf-D-Cha-l)-NH_2	
1302	TFA•H-(lvf-[*p*-F]f-l)-NH_2	
1303	TFA•H-(lvf-[F_5]f-l)-NH_2	
1306	N-methyl-(lvf-D-Cha-l)-NH_2	
1307	N-methyl-(lvf-[*p*-F]f-l)-NH_2	
1308	N-methyl-(lvf-[F_5]f-l)-NH_2	
1318	TFA•H-(lf-D-Cha-vl)-NH_2	
1319	TFA•H-(lf-[*p*-F]f-vl)-NH_2	
1320	TFA•H-(lf-[F_5]f-vl)-NH_2	
1321	2TFA•H-(lfkvl)-NH_2	
1322	TFA•H-(l-D-Cha-fvl)-NH_2	
1323	TFA•H-(l-[*p*-F]f-fvl)-NH_2	
1324	TFA•H-(l-[F_5]f-fvl)-NH_2	
1325	2TFA•H-(lkfvl)-NH_2	
1326	TFA•H-(l-D-Cha-D-Cha-vl)-NH_2	
1327	TFA•H-(l-[*p*-F]f-[*p*-F]f-vl)-NH_2	
1328	TFA•H-(l-[F_5]f-[F_5]f-vl)-NH_2	
1329	3 TFA•H-(lkkvl)-NH_2	
1125	2 TFA•H-lvf-NH-NH-fvl-H	
1133	TFA•H-lvf-NH-NH-Acetyl	
1155	TFA•H-lvf-NH-NH_2	

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. A compound comprising the structure:

$$(Y\text{-}Xaa_1\text{-}Xaa_2\text{-}NH\text{-}[(Z\text{-}Xaa_1'\text{-}Xaa_2'\text{-}Xaa_3'\text{-})\ NH]^{\diagup An}$$

wherein Xaa_1 and Xaa_2 are each D-amino acid structures and at least two of Xaa_1 and Xaa_2 are, independently, selected from the group consisting of a D-leucine structure, a D-phenylalanine structure, a D-tyrosine structure, a D-iodotyrosine structure, a D-lysine structure, or a D-valine structure;

NH-NH is a hydrazine structure;

Y, which may or may not be present, is a structure having the formula $(Xaa)_a$, wherein Xaa is any D-amino acid structure and a is an integer from 1 to 15;

Xaa_1', Xaa_2', and Xaa_3' which may or may not be present, are each D-amino acid or L-amino acid structures and at least two of Xaa_1', Xaa_2', and Xaa_3'are, independently, selected from the group consisting of a D- or L-leucine structure, a D- or L-phenylalanine structure, a D- or L-tyrosine structure, a D- or L-iodotyrosine structure, a D- or L-lysine structure, or a D- or L-valine structure;

Z, which may or may not be present, is a structure having the formula $(Xaa)_b$, wherein Xaa is any D-amino acid structure and b is an integer from 1 to 15;

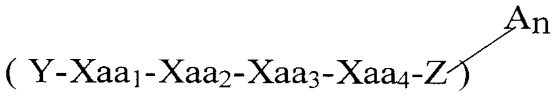
A, which may or may not be present, is a modifying group attached directly or indirectly to the compound; and

n is an integer from 1 to 15;

wherein Xaa_1, Xaa_2, Xaa_1', Xaa_2', Xaa_3', Y, Z, A and n are selected such that the compound binds to natural β-amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides, and is less prone to metabolism.

2. A compound having a structure selected from the group consisting of: H-D-Leu-D-Val-D-Phe-NH-(H-D-Leu-D-Val-D-Phe-)NH; H-D-Leu-D-Val-D-Phe-NH-NH-COCH$_3$; and H- D-Leu-D-Val-D-Phe-NH-NH$_2$.

3. A compound comprising the structure:



$$\left(Y\text{-}Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Xaa_4\text{-}Z \right)\text{—}A_n$$

wherein Xaa$_1$, Xaa$_2$, Xaa$_3$ and Xaa$_4$ are each D-amino acid structures and at least two of Xaa$_1$, Xaa$_2$, Xaa$_3$ and Xaa$_4$ are, independently, selected from the group consisting of a D-leucine structure, a D-cyclohexylalanine, a D-4-fluorophenylalanine (para-fluorophenylalanine), a D-pentafluorophenylalanine, a chlorophenylalanine, a bromophenylalanine, a nitrophenylalanine, a D-homophenylalanine, a D-lysine structure, and a D-valine structure;

Y, which may or may not be present, is a structure having the formula (Xaa)$_a$, wherein Xaa is any D-amino acid structure and a is an integer from 1 to 15;

Z, which may or may not be present, is a structure having the formula (Xaa)$_b$, wherein Xaa is any D-amino acid structure and b is an integer from 1 to 15;

A, which may or may not be present, is a modifying group attached directly or indirectly to the compound; and

n is an integer from 1 to 15;

wherein Xaa$_1$, Xaa$_2$, Xaa$_3$, Xaa$_4$, Y, Z, A and n are selected such that the compound binds to natural β-amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides, and is less prone to metabolism.

4. A compound having a structure selected from the group consisting of: N,N-dimethyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N,N-dimethyl-(D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N-methyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N-ethyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N-isopropyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Ala)-isopropylamide; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Ala)-dimethylamide; N,N-diethyl-(Gly-D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N,N-diethyl-(D-Ala-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N,N-dimethyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N,N-dimethyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N,N-dimethyl-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; H-(Gly-D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N-ethyl-(Gly-D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N-ethyl-(Gly- D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N-methyl-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; N-ethyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N-propyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; N,N-diethyl-(Gly-D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; H-(D-Ile-D-Val-D-Phe-D-Phe-D-Ile)-NH_2; H-(D-Ile-D-Val-D-Phe-D-Phe-D-Ala-)-NH_2; H-(D-Ile- D-Ile-D-Phe-D-Phe- D-Ile)-NH_2; H-(D-Nle-D-Val-D-Phe-D-Phe-D-Ala-)-NH_2; H-(D-Nle-D-Val-D-Phe-D-Phe-D-Nle)-NH_2; 1-piperidine-acetyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; 1-piperidine-acetyl-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-NH_2; H-D-Leu-D-Val-D-Phe-D-Phe-D-Leu-isopropylamide; H-D-Leu-D-Phe-D-Phe-D-Val-D-Leu-isopropylamide; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-methylamide; H-(D-Leu-D-Phe-D-Phe-D-Val-D-Leu)-methylamide; H-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-OH; N-methyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-Cha-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-[*p*-F]Phe-D-Leu)-NH_2; H-(D-Leu-D-Val-D-Phe-D-[F_5]Phe-D-Leu)-NH_2; H-(D-Leu-D-Phe-D-Cha-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Phe- D-[*p*-F]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Phe- D-[F_5]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Phe-D-Lys-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Cha-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-[*p*-F]Phe-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-[F_5]Phe-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu- D-Lys-D-Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-Cha-D-Cha-D-Val-D-Leu)-NH_2; H-(D-Leu- D-[*p*-F]Phe-D-[*p*-F]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu-D-[F_5]Phe-D-[F_5]Phe-D-Val-D-Leu)-NH_2; H-(D-Leu- D-Lys- D-Lys-D-Val-D-Leu)-NH_2; N-methyl-(D-Leu-D-Val-D-Phe-D-Cha-D-Leu)-NH_2; N-methyl-(D-Leu-D-Val-D-Phe-

D-[*p*-F]Phe-D-Leu)-NH_2; N-methyl-(D-Leu-D-Val-D-Phe-D-[F_5]Phe-D-Leu)-NH_2; H-D-Leu-D-Val-D-Phe-NH-(H-D-Leu-D-Val-D-Phe-)NH; H-D-Leu-D-Val-D-Phe-NH-NH-$COCH_3$; and H- D-Leu-D-Val-D-Phe-NH-NH_2.

5. A compound having the structure: H-(D-Leu-D-Phe-[*p*-F]D-Phe-D-Val-D-Leu)-NH_2.

6. A compound having the structure: N-methyl-(D-Leu-D-Val-D-Phe-D-Phe-D-Leu)-NH_2

7. A pharmaceutical composition comprising a therapeutically effective amount of the compound of any of claims 1, 2, 3, 4, 5, or 6 and a pharmaceutically acceptable carrier.

8. A method for inhibiting aggregation of natural β-amyloid peptides, comprising contacting the natural β-amyloid peptides with the compound of any of claims 1, 2, 3, 4, 5, or 6 such that aggregation of the natural β-amyloid peptides is inhibited.

9. A method for detecting the presence or absence of natural β-amyloid peptides in a biological sample, comprising:

contacting a biological sample with the compound of any of claims 1, 2, 3, 4, 5, or 6, wherein the compound is labeled with a detectable substance; and

detecting the compound bound to natural β-amyloid peptides to thereby detect the presence or absence of natural β-amyloid peptides in the biological sample.

10. The method of claim 9, wherein the β-amyloid modulator compound and the biological sample are contacted *in vitro*.

11. The method of claim 9, wherein the β-amyloid modulator compound is contacted with the biological sample by administering the β-amyloid modulator compound to a subject.

12. The method of claim 9, wherein the compound is labeled with radioactive technetium or radioactive iodine.

13. A method for treating a subject for a disorder associated with β-amyloidosis, comprising:

administering to the subject a therapeutically effective amount of the compound of any of claims 1, 2, 3, 4, 5, or 6 such that the subject is treated for a disorder associated with β-amyloidosis.

14. The method of claim 13, wherein the disorder is Alzheimer's disease.

				MAX SOLUBILITY								UNIT DOSE (2 mg/kg IV)			
PPI	BUI#	STRUCTURE	mwt	CONC (mg/mL)	DOSE mg/kg IV	BRAIN LEVELS (nM) individual	mean	sem	BRAIN LEVELS (nM) + CsA mean	sem		ALONE (nM)	(+) CsA (nM)	FOLD INCR. (+)CsA	PPI
		l-v-f-f-a													
558	67	4-hydroxybenzoyl-(lvffa)-NH2	714	0.8	3.3		**3.3**	1.4	**24**	5		**2**	**14**	14	558
		TFA, l-v-f-f-l													
655	75	TFA. H-(lvffl)-NH2	750	1.0	4.6	3.8, 2.6, 17.3	**7.9**	5.8	**50**	3		**3**	**22**	22	655
1019	70	TFA. N-methyl-(lvffl)-NH2	765	0.8	3.4	6.5, 7.1, 8.0	**7.2**	0.5	**29**	2		**4**	**17**	17	1019
		TFA. l-f-f-v-l													
578	75	TFA. H-(lffvl)-NH2	750	1.6	7.0	11.6, 5.7, 5.4	**7.6**	2.5	**57**	8		**2**	**16**	16	578
1320	77	TFA. H-(lf-[F$_5$]f-vl)-NH$_2$	841	0.7	3.0	4.0, 5.7, 6.3	**5.3**	0.8	**16**	2		**4**	**10**	3	1320
1324	77	TFA. H-(lf-[F$_5$]f-vl)-NH$_2$	841	1.2	4.9	1.9, 2.3, 8.2	**4.1**	2.5	**23**	5		**2**	**9**	6	1324
1318	77	TFA. H-(lf-[cha]-vl)-NH$_2$	757	0.3	1.0	3.1, 2.9, 1.5	**2.5**	0.6	**3**	0		**5**	**5**	1	1318
1319	77	TFA. H-(lf-[p-F]f-vl)-NH$_2$	769	1.7	6.6	26.4, 67.2, 11.2	**34.9**	20.5	**32**	10		**11**	**10**	1	1319
1327	77	TFA. H-(l-[p-F]f-[p-F]f-vl)-NH$_2$	787	1.0	4.0	4.9, 5.5, 5.4	**5.3**	0.2	**20**	7		**3**	**10**	4	1327
1301	77	TFA. H-(lvf-[cha]-l)-NH$_2$	757	0.7	2.9	3.5, 3.7, 2.8	**3.3**	0.4	**11**	1		**2**	**8**	3	1301
1302	77	TFA. H-(lvf-[p-F]f-l)-NH$_2$	769	0.2	0.7	2.6, 0.3, 1.2	**1.3**	0.8	**4**	1		**4**	**12**	3	1302
1328	77	TFA. H-(l-[F$_5$]f-[F$_5$]f-vl)-NH$_2$	931	0.3	1.2	1.1, 0.7, 1.4	**1.0**	0.2	**7**	1		**2**	**11**	6	1328
1322	77	TFA. H-(l-[cha]-fvl)-NH$_2$	757	0.03	0.1	0.2, 0.1, 0.3	**0.2**	0.1	**1**	0	*	**3**	**16**	5	1322
1303	77	TFA. H-(lvf-[F$_5$]f-l)-NH$_2$	841	0.3	1.0	0.6, 0.6, 1.3	**0.8**	0.3	**4**	0		**2**	**7**	5	1303
1326	77	TFA. H-(l-[cha]-[cha]-vl)-NH$_2$	763	0.05	0.2	0.6, 1.0, 1.0	**0.9**	0.2	**2**	0	*	**8**	**15**	2	1326

* COMPUTED VALUES FROM DO AREA , 100

Fig. 1

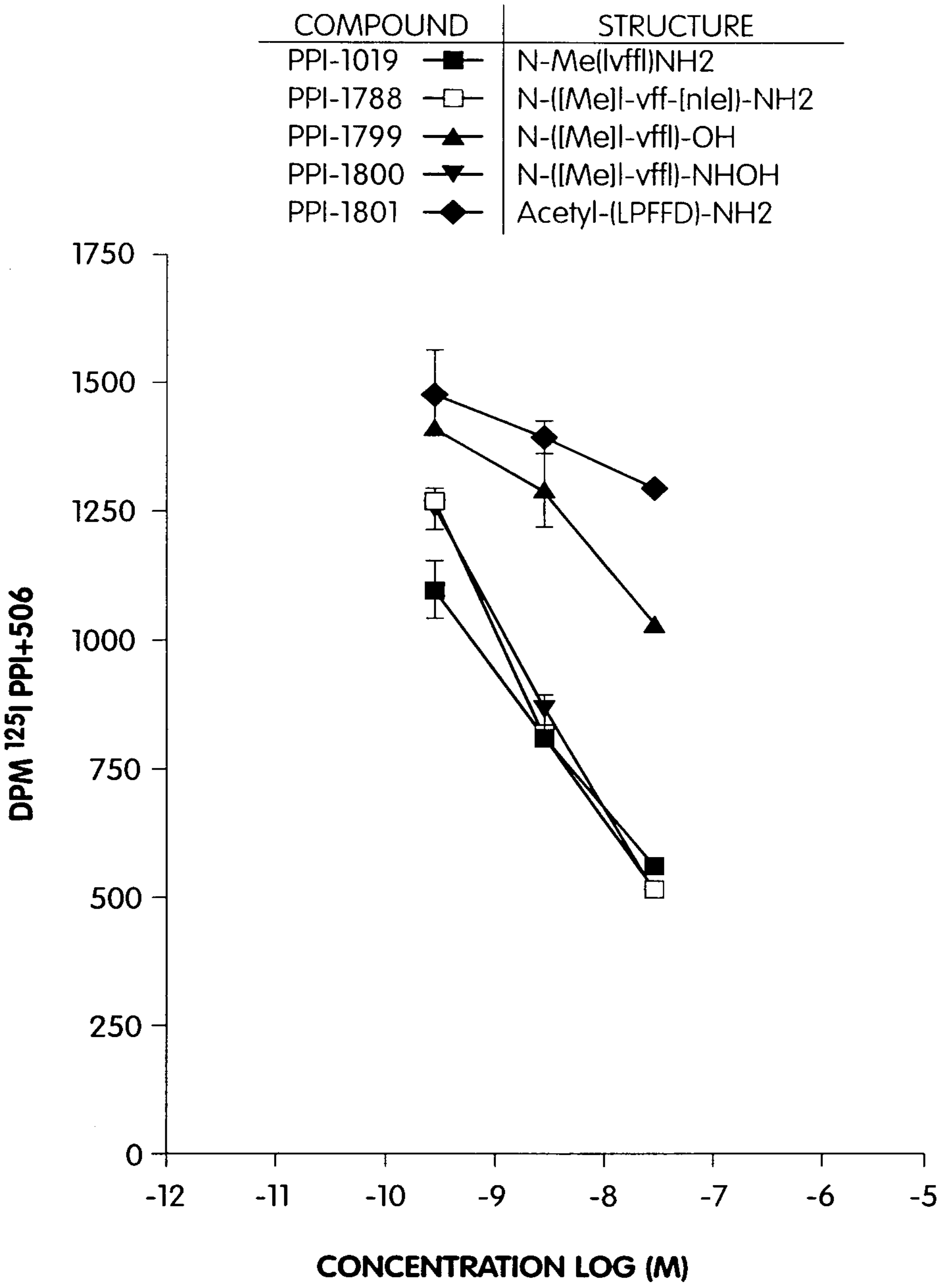

COMPOUND
STRUCTURE
PPI-1019 ■ N-Me(lvffl)NH2
PPI-1788 □ N-([Me]l-vff-[nle])-NH2
PPI-1799 ▲ N-([Me]l-vffl)-OH
PPI-1800 ▼ N-([Me]l-vffl)-NHOH
PPI-1801 ◆ Acetyl-(LPFFD)-NH2
1750
1500
1250
1000
750
500
250
0
DPM 125I PPI+506
-12
-11
-10
-9
-8
-7
-6
-5
CONCENTRATION LOG (M)

Fig. 2

INTERNATIONAL SEARCH REPORT

Inte .ional Application No
PCT/US 00/05574

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 A61K38/17 G01N33/68 A61P25/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, WPI Data, PAJ, BIOSIS, MEDLINE, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 08868 A (PRAECIS PHARM INC) 5 March 1998 (1998-03-05) the whole document ---	3-14
X	TJERNBERG L O ET AL: "CONTROLLING AMYLOID BETA-PEPTIDE FIBRIL FORMATION WITH PROTEASE-STABLE LIGANDS" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 272, no. 19, 9 May 1997 (1997-05-09), pages 12601-12605, XP002050230 ISSN: 0021-9258 See especially Fig.4 ---	3,4,7-14
A	WO 96 28471 A (PHARM PEPTIDES INC) 19 September 1996 (1996-09-19) the whole document --- -/--	1-14

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search 7 August 2000	Date of mailing of the international search report 23/08/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Groenendijk, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 00/05574

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 21728 A (KAROLINSKA INNOVATIONS AB ;NORDSTEDT CHRISTER (SE); NAESLUND JAN () 19 June 1997 (1997-06-19) the whole document ---	1-14
P,X	FINDEIS E.A.: "Modified peptide inhibitors of amyloid -beta-peptide polymerization" BIOCHEMISTRY, vol. 38, no. 21, 25 May 1999 (1999-05-25), page 6791-6800 XP002143947 EASTON, PA US see esp.page 6794, table 1 -----	3,7-14

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 00 /05574

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,2(complete),4,7-14(partially)

   Compounds having the structure defined in claim 1, their compositions and use

2. Claims: 3,5,6(complete),4,7-14(partially)

   Compounds having the structure defined in claim 3, their compositions and use

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 00 /05574

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1,3,7-14(partially)

Present claims 1 and 3 relate to peptides having 2-35 amino acid residues, lacking any constant structural domain and almost any definition of the constituting amino acid residues, which peptides are defined by reference to desirable characteristics or properties, namely that they bind to natural beta-amyloid peptides or modulate the aggregation or inhibit the neurotoxicity of natural beta-amyloid peptides when contacted with the natural beta-amyloid peptides and are less prone to metabolism.
The claims cover all compounds having these characteristics or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds defined in the claims 2,4-6.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/US 00/05574

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9808868 A	05-03-1998	AU 4238797 A	19-03-1998
		EP 0929574 A	21-07-1999
		US 5985242 A	16-11-1999
WO 9628471 A	19-09-1996	US 5817626 A	06-10-1998
		US 5854215 A	29-12-1998
		AU 5252496 A	02-10-1996
		CA 2214247 A	19-09-1996
		EP 0815134 A	07-01-1998
		JP 11514333 T	07-12-1999
		US 5854204 A	29-12-1998
		US 5985242 A	16-11-1999
WO 9721728 A	19-06-1997	AU 1072897 A	03-07-1997
		EP 0866805 A	30-09-1998

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{7} **:**
C07K 14/47, A61K 38/17, G01N 33/68, A61P 25/28, C12N 5/00, A61K 51/00

A2

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60/132,592 5 May 1999 (05.05.99) US

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(72) Inventors; and
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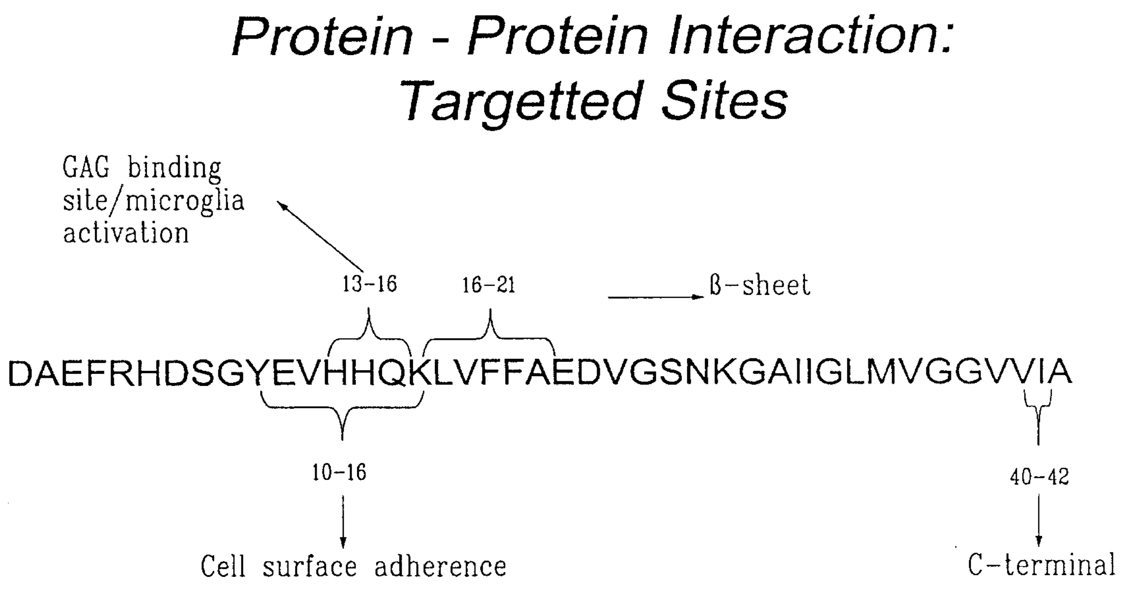
(74) Agents: MURPHY, Kevin, P. et al.; Swabey Ogilvy Renault, Suite 1600, 1981 Mcgill College Avenue, Montréal, Québec H3A 2Y3 (CA).

(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: STEREOSELECTIVE ANTIFIBRILLOGENIC PEPTIDES AND PEPTIDOMIMETICS THEREOF


Protein - Protein Interaction:
Targetted Sites
GAG binding site/microglia activation
13-16
16-21
ß-sheet
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
10-16
Cell surface adherence
40-42
C-terminal

(57) Abstract

The present invention relates to antifibrillogenic agents for inhibiting amyloidosis and/or for cytoprotection for the treatment of amyloidosis disorders. These agents include peptides, isomers thereof and peptidomimetic compounds thereof. These agents comprise a peptide having a sequence identified from the glycosaminoglycan (GAG) binding region and the prot–prot interaction region of the amyloid protein. The peptide has at least one [D] amino acid isomer substitution. The invention also relates to the peptide bound to a label for *in vivo* imaging of amyloid deposits.

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STEREOSELECTIVE ANTIFIBRILLOGENIC PEPTIDES AND PEPTIDOMIMETICS THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to agents having potent antifibrillogenic activity for the treatment of amyloidosis disorders and for imaging of amyloid deposits. These agents include peptides and peptidomimetic compounds thereof.

(b) Description of Prior Art

Amyloidosis refers to a pathological condition characterized by the presence of amyloid fibers. Amyloid is a generic term referring to a group of diverse but specific extracellular protein deposits that are seen in a number of different diseases. Though diverse in their occurrence, all amyloid deposits share common morphologic properties, stain with specific dyes (e.g. Congo red), and have a characteristic red-green birefringent appearance in polarized light after staining. They also share common ultrastructural, x-ray diffraction and infrared spectra features.

Some amyloidotic diseases can be idiopathic but most of these diseases appear as a complication of a previously existing disorder. For example, primary amyloidosis can appear without any other pathology or can follow plasma cell dyscrasia or multiple myeloma. Secondary amyloidosis is usually seen associated with chronic infection (such as tuberculosis) or chronic inflammation (such as rheumatoid arthritis). A familial form of secondary amyloidosis is also seen in Familial Mediterranean Fever (FMF). This familial type of amyloidosis, as one of the other types of familial amyloidosis, is genetically inherited and is found in specific population groups. Isolated forms of amyloidosis are those that tend to involve a single organ system. Different amyloids are also characterized by the type of protein present in the deposit. For example, neurodegenerative diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease and the like are characterized by the appearance and accumulation of a protease-resistant form of a prion protein (referred to as AScr or PrP-27) in the central nervous system. Similarly, Alzheimer's disease, another neurodegenerative disorder, is characterized by congophilic cerebral angiopathy, neuritic plaques and neurofibrillary tangles. In this case, the plaque and blood vessel amyloid is formed by the deposition of fibrillar Aβ amyloid protein. In adult-onset diabetes, amyloids containing the IAPP amyloid protein accumulate in the pancreas. Other systemic diseases, complications of long-term hemodialysis and sequelae of long-standing inflammation or plasma cell dyscrasias are characterized by the accumulation of amyloids systemically. In each of these cases, a different amyloidogenic protein is involved in amyloid deposition.

Once these amyloids have formed, there is no known, widely accepted therapy or treatment that significantly dissolves the deposits *in situ*.

Each amyloidogenic protein has the ability to organize into β-sheet and to form insoluble fibrils that get deposited extracellularly. Each amyloidogenic protein, although different in amino acid sequence has the same property of forming fibrils and binding to other elements such as proteoglycan (glycosaminoglycan), amyloid P and complement component. Moreover, each amyloidogenic protein has amino acid sequences which, although different, will show similarities such as regions with the ability to bind to GAG's (referred to as the GAG binding site) as well as other regions which will promote β-sheet formation referred to as β-sheet region.

In specific cases, amyloidotic fibrils once deposited can become toxic to the surrounding cells. As per example, the Aβ fibrils organized as senile plaques have been shown to be associated with dead neuronal cells and microgliosis in patients with Alzheimer's disease. When tested *in vitro*, Aβ peptide was shown to be capable of triggering an activation process of the microglia (brain macrophages), which would explain the presence of microgliosis and brain inflammation found in the brain of patients with Alzheimer's disease.

In another type of amyloidosis seen in patients with Type II diabetes, the amyloidogenic protein IAPP, has been shown to induce β-islet cell toxicity *in vitro*. Hence, appearance of IAPP fibrils in the pancreas of Type II diabetic patients could contribute to the loss of the β islet cells (Langerhans) and organ dysfunction.

Particularly, in patients with Alzheimer's Disease, an agent capable 1) of preventing amyloid fibril formation and deposition and 2) of directly or indirectly inhibiting Aβ-induced neurotoxicity and inflammation (microgliosis), could be a treatment of choice to prevent and arrest the development of Alzheimer's disease.

It would be highly desirable to be provided with agents having potent antifibrillogenic activity for the treatment of amyloidosis disorders.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide agents having potent antifibrillogenic activity for the treatment of amyloidosis disorders.

Another aim of the present invention is to provide a method for the treatment of amyloidosis disorders, such as Alzheimer's' disease.

A number of strategies for possible therapeutic intervention in amyloid development have been proposed. These strategies include reduction of the pool of precursor proteins, prevention of the interaction of precursor proteins and disruption of preformed amyloid. The present invention deals mainly with the second approach, prevention of precursor protein interactions. The ideal molecule to fulfill this function, would interact specifically with the amyloid protein and would in so doing prevent the protein from interacting with itself. When dealing with molecules that are chiral, it is standard practice to identify which of the stereoisomers possesses the activity, since in general, activity can be attributed to one or the other of the isomers. By using a stereochemically pure isomer, side reactions can be avoided or reduced.

In accordance with one embodiment of the present invention there is provided an antifibrillogenic agent for inhibiting amyloidosis and/or for cytoprotection, which comprises a peptide of Formula I, an isomer thereof, a retro or a retro-inverso isomer thereof or a peptidomimetic thereof:

$$Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Xaa_4 \qquad \text{I}$$

wherein,

Xaa_1 is absent or selected from the group consisting of Lys, Lys-Lys, Xaa_5-Lys-, and Ala;

Xaa_5 is absent or selected from the group consisting of His-Gln-, His-His-Gln-, Val-His-His-Gln-, Glu-Val-His-His-Gln-, Asp-Asp-Asp-, Lys-Val-Asp-Asp-Gln-Asp-, Gln-;
Xaa_2 is absent or any amino acid;
Xaa_3 is absent, Val or Phe;
Xaa_4 is absent or selected from the group consisting of Phe, Phe-NH_2, Phe-Phe, Phe-Phe-Ala, Phe-Phe-Ala-NH_2, Phe-Phe-Ala-Gln, Phe-Phe-Ala-Gln-NH_2, Val-Leu-Lys, Val-Leu-Lys-NH_2;
wherein the peptide of formula I contains at least one Lys or Asp;
and wherein the peptide has at least one [D] amino acid residue,
with the proviso that Lys-Lys-Leu-Val-Phe-Phe-Ala is an all-[D] peptide; and with the proviso that when Xaa_5 is Lys-Val-Asp-Asp-Gln-Asp- all of Xaa_2, Xaa_3, and Xaa_4 are absent.

Preferably, Xaa_2 is a hydrophobic amino acid residue such as a leucine residue.

In one embodiment of the invention, the peptide of formula I has at least two [D] amino acid residues, and more preferably at least three [D] amino acid residues. Optionally, the peptide of formula I has one [L] amino acid residue, or more preferably the peptide is an all-[D] isomer peptide.

In another embodiment of the invention, the peptide of Formula I is selected from the group consisting of:

Lys-Ile-Val-Phe-Phe-Ala	(SEQ ID NO:1);
Lys-Lys-Leu-Val-Phe-Phe-Ala	(SEQ ID NO:2);
Lys-Leu-Val-Phe-Phe-Ala	(SEQ ID NO:3);
Lys-Phe-Val-Phe-Phe-Ala	(SEQ ID NO:4);
Ala-Phe-Phe-Val-Leu-Lys	(SEQ ID NO:5);
Lys-Leu-Val-Phe	(SEQ ID NO:6);
Lys-Ala-Val-Phe-Phe-Ala	(SEQ ID NO:7);

Lys-Leu-Val-Phe-Phe (SEQ ID NO:8);
Lys-Val-Val-Phe-Phe-Ala (SEQ ID NO:9);
Lys-Ile-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:10);
Lys-Leu-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:11);
Lys-Phe-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:12);
Ala-Phe-Phe-Val-Leu-Lys-NH_2 (SEQ ID NO:13);
Lys-Leu-Val-Phe-NH_2 (SEQ ID NO:14);
Lys-Ala-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:15);
Lys-Leu-Val-Phe-Phe-NH_2 (SEQ ID NO:16);
Lys-Val-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:17);
Lys-Leu-Val-Phe-Phe-Ala-Gln (SEQ ID NO:18);
Lys-Leu-Val-Phe-Phe-Ala-Gln-NH_2 (SEQ ID NO:19);
His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:20);
Asp-Asp-Asp (SEQ ID NO:21);
Lys-Val-Asp-Asp-Gln-Asp (SEQ ID NO:22);
His-His-Gln-Lys (SEQ ID NO:23);
and
Gln-Lys-Leu-Val-Phe-Phe-NH_2 (SEQ ID NO:24).

More preferably, the peptide of formula I is a peptide as set forth in SEQ ID NO:2 or SEQ ID NO:3.

In accordance with one embodiment of the present invention there is provided a labeled conjugate for *in vivo* imaging of amyloid plaque, which comprises a conjugate of formula II:

$$A\text{-}B\text{-}C \qquad \text{II}$$

wherein A is an amyloid plaque-targeting moiety selected from the group consisting of a peptide of Formula I as defined above, an isomer thereof, a retro or a retro-inverso isomer thereof and a peptidomimetic thereof,
wherein B is a linker portion allowing attachment of the amyloid plaque-targeting moiety to C; and
wherein C is a label that allows for *in vivo* imaging.

Preferably, the linker portion B is selected from the group consisting of Glucose and Phe. Preferably, the label C is ^{99m}Tc.

Still in accordance with the present invention, there is provided a method for the treatment of amyloidosis disorders in a patient, which comprises administering to the patient a therapeutically effective amount of a peptide of Formula I, or the antifibrillogenic agent, as defined above.

Further in accordance with the present invention, there is provided a composition for the treatment of amyloidosis disorders in a patient, which comprises a therapeutically effective amount of a peptide of Formula I, or of an antifibrillogenic agent, as defined above in association with a pharmaceutically acceptable carrier.

In accordance with the present invention, there is also provided a composition for *in vivo* imaging of amyloid plaques, which comprises a therapeutically effective amount of a labeled conjugate as defined above in association with a pharmaceutically acceptable carrier.

The peptide of Formula I or the antifibrillogenic agent may be used for inhibiting amyloidosis and/or for cytoprotection.

The labeled conjugate may be used for *in vivo* imaging of amyloid plaques.

The peptide of Formula I or the antifibrillogenic agent may alternatively be used for the manufacture of a medicament for inhibiting amyloidosis and/or for cytoprotection.

Similarly, the labeled conjugate may also be used for the manufacture of a medicament for *in vivo* imaging of amyloid plaques.

Other embodiments of these peptides include racemic mixtures and peptides having mixed chirality, i.e., different chirality at different chiral centers.

In accordance with the peptides Lys-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:2) and Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:3), one stereoisomer, the D form, is found to be more active than the L form, and the D isomer is the preferred form for use of this peptide as a drug.

The present invention further provides similar peptides designed for the other amyloidogenic peptides such as AA, AL, and IAPP. In fact, the present invention also provides a peptide for inhibiting amyloidosis and/or for cytoprotection. The peptide has a sequence taken from the β-sheet region of an amyloid protein. Such peptide or a composition containing such peptide can be used for inhibiting amyloidosis and/or for cytoprotection. Alternatively, such peptide or a composition containing such peptide can be used for the manufacture of a medicament for inhibiting amyloidosis and/or for cytoprotection.

Accordingly, the present invention also provides a composition for inhibiting amyloidosis and/or for cytoprotection, which comprises a therapeutically effective amount of a peptide as defined previously in association with a pharmaceutically acceptable carrier.

In accordance with the present invention, the amyloidosis disorder includes, without limitation, prion protein related disorders, type II diabetes and Alzheimer's disease.

With regard to another aspect of the invention, diseases caused by the death or malfunctioning of a particular type or types of cells can be treated by transplanting into the patient healthy cells of the relevant type of cell. Often these cells are cultured *in vitro* prior to transplantation to increase their numbers, to allow them to recover after the isolation procedure or to reduce their immunogenicity. However, in many instances the transplants are unsuccessful, due to the death of the transplanted cells. The inventors have now also found that culturing of cells can lead to the formation of fibrils from endogenous proteins. Such fibrils are likely to continue to grow after the cells are transplanted and cause death or dysfunction of the cells. The inventors have also found that the peptide of the present invention or the antifibrillogenic compound of the present invention can be used to reduce the formation of fibrils.

Thus the invention also provides a process for the preparation of cells suitable for transplantation into a mammal, which cells are capable of forming fibrils. The process comprises contacting the cells with the peptide of the present invention or the antifibrillogenic compound of the present invention.

The peptide of Formula I or the antifibrillogenic compound causes breakdown of amyloid deposits which have been formed by the cells prior to the contact. Preferably, the cells are cultured in the presence of the peptide of Formula I or the antifibrillogenic compound.

For the purpose of the present invention the following expressions and terms are defined below.

The term "agents having stereoselective antifibrillogenic activity" is intended to mean any peptides, peptide analogues, peptide derivatives, or peptidomimetics which retain the stereoselective antifibrillogenic activity, the cytoprotective and anti-inflammatory activity and/or the ability to alter a natural amyloidotic protein aggregation as described herein. Peptide analogues, peptide derivatives, or peptidomimetics include any molecules that mimic the chemical structure of a peptide and retain the functional properties of the peptide (Williams, W.V. and Weiner, D.B., eds., Biologically Active Peptides: Design, Synthesis, and Utilization, vol. 1, Technomic Publishing Company Inc., Lancaster, Pa., 1993, pages 35-3..). Examples of peptide analogues, peptide derivatives, or peptidomimetics include compounds with sulfonamide, phosphoramide or non-amide linkages.

The expression "antifibrillogenic activity" is intended to mean the ability to block or prevent an amyloidogenic protein from forming fibrils, preferably by preventing it from adopting its β-pleated conformation.

The term "cytoprotection" or "cytoprotective activity" is intended to mean the ability to protect cells from amyloid-induced toxicity.

The expression "anti-inflammatory" is intended to mean the ability to block or reduce the Aβ-induced microglial activation process or to block the chemokine-induced inflammatory reaction.

The expression "retro isomer" is intended to mean a reversal of the direction of the peptide backbone.

The expression "inverso isomer" is intended to mean an inversion of the amino acid chirality used to make the peptide.

The expression "retro-inverso isomer" is intended to mean a reversal of both the peptide backbone direction and the amino acid chirality.

Except as otherwise expressly defined herein, the abbreviations used herein for designating the amino acids and the protective groups are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry*, 1972, **11**:1726-1732).

Also, unless specified otherwise, the Aβ(1-40) is the naturally occurring Aβ(1-40), that is the all [L]-isomer.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the targeted sites of the protein-protein interactions required for self-assembly into β-sheet fibrils;

Fig. 2 illustrates a thioflavin T fluorescence assay for fibril formation by [L]-Aβ (1-40) in the absence and presence of a peptide in accordance with one embodiment of the invention;

Fig. 3 shows the same assay as in Fig. 2 for fibril formation by [D]-Aβ (1-40);

Fig. 4 is a bar graph illustrating the percentage of thioflavin T fluorescence in the presence of the [D]-peptide used in Fig. 2, with or without single substitutions of corresponding [L]-amino acids;

Fig. 5 is a bar graph illustrating a thioflavin T fluorescence assay for fibril formation by [L]-Aβ (1-40) in the presence of the [D]-peptide used in Fig. 2, with or without substitution of the Leu residue by other hydrophobic amino acids;

Fig. 6 illustrates the toxicity of [L]-Aβ (1-40) in the absence and presence of peptides in accordance with one embodiment of the invention; and

Fig. 7 is a bar graph illustrating the toxicity of [L]-Aβ (1-40) in the presence of another peptide of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

As illustrated in Fig. 1, internal regions of the Aβ sequence have been shown to confer characteristics of the amyloid protein. Indeed, the region between amino acid 13-16 (His-His-Gln-Lys, SEQ ID NO:23) of the amyloid protein is responsible for the interaction between the Aβ protein and the glycosaminoglycan moiety of the proteoglycans (Kisilevsky, R., et al., Proteoglycans and amyloid fibrillogenesis: The nature and origin of amyloid fibrils, Wiley, Chichester (*CIBA Foundation Symposium 1997*), pp. 58-72). Proteoglycans are known to promote amyloid fibril formation as well as protect these fibrils from proteolysis (Gupta-Bansal, R., et al., 1995, *The Journal of Biological Chemistry*, **270**:18666-18671). More recently, the same region has been determined to play a role in the activation process of microglial cells by Aβ (Giulian, D., et al., 1998, *The Journal of Biological Chemistry*, **273**(45):29719-29726). This 13-16 region of Aβ, often referred to as the GAG binding site, is also part of a larger domain, the 10-16 region of the protein which has been suggested as the region responsible for the adherence of Aβ to the cell surface (Giulian, D., et al., 1996, *The Journal of Neuroscience*, **16**(19):6021-6037). Such adherence of Aβ to the cell surface will allow the interaction of Aβ with the specific cells leading to either microglia activation or toxicity of neuronal cells.

These two overlapping regions of the Aβ protein, i.e. amino acids 13-16 and 10-16 are adjacent to the 16-21 region of Aβ, a short hydrophobic stretch critical for the formation of fibrillar structures (Hilbrich, C., et al., 1992, *J. Mol. Biol.*, **228**:460-473). By having peptides capable of interacting with these overlapping regions of Aβ, one can aim at preventing both Aβ fibril formation and Aβ cellular interaction (i.e. microglia activation, neurotoxicity).

A preferred embodiment of the present invention is novel and arises from the unexpected finding that the all-[D] stereoisomer peptides, Lys-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:2) and Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:3), are much more potent inhibitors of Aβ(1-40) fibrillogenesis then the corresponding all-[L] peptides. The all-[D] stereoisomer peptides, Lys-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:2) and Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:3) are also potent cytoprotective agents.

This finding was unforeseen particularly because the researchers who originally reported peptides containing the sequence Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:3) as an inhibitor of fibrillogenesis, state in a second article which they published: "A peptide entirely composed of amino acids in D configuration with the sequence klvff (lowercase marks amino acids in D configuration) was synthesized using the SPOT technique and assayed for ^{125}I-LBMP1620 binding. This peptide failed to bind ^{125}I-LBMP1620 indicating that KLVFF-KLVFF interaction is sterospecific." Tjernberg, L.O. et al. (1997) Controlling Amyloid β-Peptide Fibril Formation with Protease-stable Ligands, *J. Biol. Chem.*, **272**:12602.

Inhibition of Amyloidosis

The experimental work performed leading to this invention included comparing the ability of the [D] and [L] stereoisomers of peptide Lys-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:2) to inhibit the fibrillogenesis process observed with the amyloidogenic peptide Aβ(1-40) in a thioflavin T fluorescence assay.

The thioflavin T fluorescence assay for fibrillogenesis is based on the principle that the fluorescent dye, thioflavin T, binds specifically to fibrillar, but not to unaggregated Aβ peptide (LeVine III, H., 1993, *Protein Science* **2**:404-410). Upon binding, thioflavin T develops a characteristic fluorescence (Naiki, H., et al., 1996, *Lab. Invest.* **74**: 374-383) which can be easily detected. The dye is believed to interact with the stacked cross-β pleated sheets, the common structural motif of all amyloids (LeVine III, H., 1995, *Amyloid: Int. J. Exp. Clin Invest.* **2**:1.6). Thioflavin T is widely used to assay the effect of compounds on Aβ peptide fibrillogenesis (Bronfman, P.C., et al., 1995, *Neuroscience Lett.* **218**:201-203).

In this assay test compounds are incubated with a solution of Aβ(1-40) (20 μM) containing 10 μM thioflavin T, in 0.02M Tris/0.02M acetate/0.15M NaCl/0.005% azide/pH 7.40 at 37°C in sealed 384 well microplates. Readings (ex 430 nm/em 485nm) are taken at various time intervals with a microplate fluorescence reader. An increase in fluorescence signifies the appearance of amyloid or intermediates in the production of amyloid. Inhibitors of fibrillogenesis will lead to less fluorescence production.

The results illustrated in Table 1 below, are based on the fluorescence production in the presence of test peptides at either 20 μM or 80 μM concentration, at the time intervals of 5, 19, 45, 67, 77 and 90 hours, compared to a control, buffer alone, without added inhibitory peptide.

Table 1

Order Of Potency of Peptide Inhibitors

	Tested at 20μM	Tested at 80μM
(strongest activity)	1 (D) KIVFFA	1 (D) AFFVLK
	2 (D) KKLVFFA	1 (D) KKLVFFA
	3 (D) KLVFFA	1 (D) KLVFFA
	4 (D) KFVFFA	1 (D) KFVFFA
	5 (D) AFFVLK	5 (D) KIVFFA
	6 (D) KLVF	6 (D) KAVFFA
	7 (D) KAVFFA	7 (L) KKLVFFA
	8 (L) KLVFFA	8 (L) KLVFFA
	9 (D) KLVFF	9 (D) KLVF
	10 (L) KKLVFFA	10 (D) KLVFF
(weakest activity)	11 (L) AFFVLK	11 (L) AFFVLK

Protocol

<u>Aβ peptide</u>: Aβ(1-40) 95% purity (American Peptide Company, Inc, Sunnyvale, Cal. USA, cat. 62-0-78) is disaggregated in trifluoroacetic acid and filtered through a 0.02 μM filter, (Whatman Anotop 25 plus, .02 μm, Catalogue no. 6809 4102) in hexafluoroisopropanol (HFIP). Solutions of Aβ(1-40) at 600 μM in HFIP are stored at -80°C.

<u>Assay mixture</u>: The mixture is prepared as two solutions that are combined upon addition to the 384 well microplate (Corning Costar cat. 3705).

i) Solution A consists of test peptides in 0.02M Tris/0.02M acetate/0.15M NaCl/0.01 % azide at pH 7.40 or buffer alone (control),

ii) Solution B consists of Aβ(1-40) 40 μM, thioflavin T 20 μM in 0.02M Tris/0.02M acetate/0.15M NaCl at pH 7.40. This solution is prepared by drying the Aβ peptide under nitrogen and then resuspending this in 0.04M Tris base with 15 minutes sonication. An equal volume of 0.04M acetic acid containing 0.3 M NaCl is added and the solution is adjusted to pH 7.40±0.02. A small volume of 5mM thioflavin T is added to the solution to give a final 20 μM concentration of thioflavin T.

iii) The microplate is loaded with 40 μL of solution A followed by 40 μL of solution B which gives a final 20 μM Aβ(1 -40), 10 μM thioflavin T, and either 20 μM, 80 μM or 100 μM test compound in 0.02M Tris/0.02M acetate/0.15M NaCl/0.005% azide, pH 7.40. The plate is sealed and loaded into the microplate fluorescence reader.

Fluorescence measurement data analysis: The HTS-7000 Bio Assay Reader, Perkin Elmer, is used to perform kinetic runs of about 5 days. Readings were taken at various time intervals, 5, 19, 45, 67, 77 and 90 hours, with one minute shaking before each reading. Bandpass filters used were: excitation 430 nm, emission 485 mm.

Calculations

The rank order of efficacy of the peptides is determined by observing which peptides allow the appearance of fluorescence, above the background level, first. For example in the presence of buffer control alone, fluorescence appears earlier than when any of the peptides is present. The most active peptides prevent the appearance of fluorescence even after 90 hours of incubation.

The results achieved in the thioflavin T fibrillogenesis assays show that all-[D] stereoisomer peptide was about 60 times more potent then the all-[L] stereoisomer peptide. This is based on the observation that 400 μM all-[L] stereoisomer was required to give an equivalent inhibition to that produced with 6.1 μM all-[D] stereoisomer peptide.

The results achieved in the Aβ-NBD environmental probe fibrillogenesis assay showed that the all-[D] stereoisomer peptide was at least 30 times more potent than the all-[L] stereoisomer peptide. This estimate is based on the observation that the lowest concentration of all-[D] peptide tested (25 μM) was more potent than the highest concentration of the all-[L] peptide (800 μM).

β-sheet and GAG binding domains peptides

Novel peptides and peptidomimetics that include complementary sequences to certain portions of amyloidogenic peptides such as Aβ, AA, AL, IAPP, and prion proteins are designed to be capable of inhibition of Protein-Protein interactions or self assembly. The targeted portions in the various disease-causing proteins aforementioned, preferably contain one or more charged residues such as aspartate, glutamate, lysine, histidine and arginine. Such peptides and their peptidomimetics will inhibit fibrillogenesis of the amyloidogenic peptides and prion proteins and interfere with chemokines binding to the cell surface proteoglycans leading to dimerization or tetramerization by interacting with their GAG binding domains. In the case of Aβ, these interactions lead to cytoprotection as well as inhibition of inflammatory response and serve as potent therapeutics for the treatment of Alzheimer's disease. In the case of chemokine-related disorders these interactions may lead to a decrease in the uncontrolled inflammatory response associated with some diseases.

Other amyloidogenic peptides such as IAPP, have also been tested. For example, 2 peptides from the β-sheet region of IAPP have been shown to inhibit IAPP fibril formation using the thioflavin T fluorescence assay, circular dichroism (measures secondary structure) and the electron microscope (to look at fibrils directly).

The full-length IAPP is 37 amino acids and the β-sheet region is the 20-29 sequence. The 20-29 sequence is critical for forming β-sheet and has been previously shown to be a key region in modulating IAPP aggregation and folding. Hexapeptides from this β-sheet region were examined and 2 were found to be active.

Hexapeptides spanning the 20-29 region (Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser) of the IAPP protein were synthesized and tested for their ability to prevent fibril formation as determined by circular dichroism and the thioflavin T assay. Hexapeptides were designed and were found to be capable of blocking the formation of IAPP fibrils. These peptides (Ser-Asn-Asn-Phe-Gly-Ala- and Asn-Asn-Phe-Gly-Ala-Ile) were directed towards the central core of the 20-29 region.

Novel peptides containing 3-6 residues that are complementary (in terms of their charges) to the 10-16 segment of Aβ peptide have been shown for the first time to strongly interact with Aβ peptide. They provide a starting point for the design of BBB (blood brain barrier) permeable peptidomimetics. In principle, the present invention provides similar peptides can be designed for the other amyloidogenic peptides such as AA, AL, and IAPP.

Asp-Asp-Asp (SEQ ID NO:21), a tripeptide, when incubated with Aβ40 under physiological conditions shows a slight decrease at time t=0 in the amount of β-sheet content as is evident by the CD spectrum. Incubation of this tripeptide with Aβ40 for 24 hours shows no trace of β-sheet conformation of the Aβ40 and clearly indicates the ability of this tripeptide to strongly interact with Aβ40 peptide and keep Aβ40 in a randomized and non-fibrillary conformation. The anti-fibrillogenic property of this tripeptide is also supported by the Aβ42 solubilization assay.

Lys-Val-Asp-Asp-Gln-Asp (SEQ ID NO:22), a hexapeptide, when incubated with Aβ40 under physiological conditions shows an increase at time t=0 in the amount of β-sheet content as is evident by the CD spectrum. Incubation of this hexapeptide with Aβ40 for 24 hours shows a dramatic increase in β-sheet content of the Aβ40 and clearly indicates the ability of this hexapeptide to strongly interact with Aβ40 peptide and organize it into a β-sheet conformation. Electron microscopy of the mixture failed to show any fibrils indicating that this particular compound is in fact an anti-fibrillogenic compound with regard to Aβ. *In vitro* results with NBD and thioflavin-T based fluorescence assays confirm this finding. It is the understanding of the inventors that this interesting observation will lead to a greater understanding of fibrillogenesis of Aβ40 and Aβ42 peptides and as a result, will provide important information for the design of potent anti-fibrillogenic compounds for Aβ, other amyloidotic peptides such as AA, AL and IAPP for the treatment of diseases such as Alzheimer's, Type II Diabetes and amyloidosis related disorders. The same principle can also be applied to the design of peptide type compounds for the inhibition of binding of various chemokines to the cell surface as well as inhibition of self assembly and cellular adherence of prion proteins.

The results illustrated in Fig. 2 show that all [D]-Lys-Leu-Val-Phe-Phe-Ala (SEQ. ID NO: 3) is a more potent inhibitor of Aβ (1-40) assembly in the thioflavin T fluorescence assay than is all [L]-Lys-

Leu-Val-Phe-Phe-Ala. Since the naturally occurring Aβ (1-40) used in these experiments was the all-[L] amino acid version, these results indicate that an inhibitor peptide works best when containing amino acids of the opposite chirality.

Fig. 3 demonstrates that the same rule of opposite chirality illustrated in Fig. 2 applies for the assembly of Aβ (1-40) synthesized using amino acids of the [D] type. In this experiment all-[L]-Lys-Leu-Val-Phe-Phe-Ala (SEQ. ID NO:3) is a more potent inhibitor in the all-[D]-Aβ (1-40) assembly reaction than all-[D]-Lys-Leu-Val-Phe-Phe-Ala. This result confirms that peptides of opposite chirality are better inhibitors.

Fig. 4 illustrates the inhibition of Aβ (1-40) fibril formation by all-[D]-Lys-Leu-Val-Phe-Phe-Ala (20μM) with or without single substitutions of [L]-amino acids in the thioflavin T fluorescence assay. In this experiment the ability of the all-[D]-Lys-Leu-Val-Phe-Phe-Ala peptide to inhibit Aβ (1-40) fibril formation, measured as percentage of thioflavin T fluorescence in the absence of peptide (control), was compared to [D]-Lys-Leu-Val-Phe-Phe-Ala peptides with single [L]-amino acid replacements. None of the mixed chirality Lys-Leu-Val-Phe-Phe-Ala peptides were more potent than the original all-[D] peptide. This result demonstrates that [D]-amino acids are more potent inhibitors of Aβ (1-40) fibrillogenesis than [L]-amino acids.

However as seen in Fig. 4 some peptides with single [L] substitutions do retain inhibitory activity. In particular peptides in which the [D] isomer of the Lys, the second Phe and the Ala are substituted with the [L]-isomers retain inhibitory activity. The substitutions, which reduce inhibitory activity the most, are the Leu, the Val and the first Phe, indicating that these residues contribute the most to the potency of the [D]-peptide. From Fig. 4, it is apparent that peptides with mixed chirality or with at least one [D]-substituted amino acid are also inhibitors, although not as potent as the all-[D] peptide. These mixed-chirality peptides are thus meant to be included in the present invention.

Fig. 5 illustrates the inhibition of Aβ (1-40) fibril formation in the thioflavin T fluorescence assay by all-[D]-Lys-Leu-Val-Phe-Phe-Ala (20μM), with or without replacement of the leucine by other hydrophobic amino acids. In this experiment all the peptides tested retained some inhibitory activity. This result demonstrates that the leucine residue is not critical for inhibition of Aβ fibril formation in the all-[D] peptide. These results illustrated in Fig. 5 were non-obvious and unexpected in light of a prior publication which identified the Leucine residue as critical in an all-[L] version of the peptide (Tjernberg LO et al., *J. Biol. Chem.* **271**:8545, 1996).

Cytoprotection

The experimental work performed leading to this invention also included comparing the ability of [D] and [L] stereoisomers of the peptides of the present invention to show cytoprotective activity, i.e. to protect cells from Aβ toxicity.

Figure 6 uses the MTT assay on SH-SY5Y cells.

Protocol

A SH-SY5Y human neuroblast cell line (American Type Culture Collection, cat. CRL-2266) is cultured according to technical specifications. Monomerized Aβ (1-40) is prepared using trifluoroacetic acid and hexafluoroisopropanol, in the same way already described for the thioflavin T fluorescence assay.

Monomerized Aβ at various concentrations in TANA buffer (0.02 M TRIS base pH 7.4, 0.02M acetate, 0.15 M NaCl) is added to 100 μM test peptide and the mixture is incubated for 24 hours at 37°C with agitation, in order to allow polymerization to occur. Cells are adhered to a 96-well microplate for 2 hours at 37°C and 5% CO_2 before the Aβ-peptide mixture, or buffer alone (control), is added. The microplate is gently agitated and incubated for 20-24 hours at 37°C and 5% CO_2. Cell viability is determined by a MTT-based colorimetric assay. The MTT assay (Boehringer Mannheim, Cell Proliferation Kit 1) is based on the principle that the yellow tetrazolium salt MTT is cleaved in metabolically-active cells to produce purple formazan crystals. The formazan crystals are solubilized and the resulting colored solution is quantified using a scanning multiwell spectrophotometer (ELISA reader, Absorbance A_{560} nm). Cellular toxicity was calculated as follows:

$$\text{Toxicity (\%)} = 100 - \frac{(\text{O.D. sample} - \text{O.D. Blank})}{(\text{O.D. Control} - \text{O.D. Blank})}.$$

Figure 6 shows the neurotoxicity of Aβ (1-40) in the absence or presence of various peptides of the present invention. In this experiment the all-[D]-Lys-Lys-Leu-Val-Phe-Phe-Ala (SEQ. ID NO: 2) peptide is a more potent inhibitor of Aβ neurotoxicity than the all-[L]-Lys-Lys-Leu-Val-Phe-Phe-Ala peptide in the cytoprotection assay.

Figure 7 uses the propidium iodide assay on primary cortical neurons. Briefly, fetal rat primary cortical neurons are isolated and cultured according to Durkin, J.P. et al., J. Neurochem., 66:951-962, 1996. Neurons are plated in a 48 well microplate. 7 days after plating the neuronal culture media is supplemented with B27 (Life Technologies, Data sheet form No. 3755). A mixture of Aβ and test peptide is added to the cortical neurons for 3 days at 37°C and 5% CO2.

Cell viability is then visually assessed as the proportion of phase-bright cells that exclude propidium iodide, since only dead cells take up propidium iodide.

Figure 7 shows the potent cytoprotective activity of all-[D]-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:3). This experiment shows the potent cytoprotective activity of all-[D]-Lys-Leu-Val-Phe-Phe-Ala compared to Congo red, which is a known cytoprotective agent and compared to the absence of any cytoprotective agent (Aβ alone).

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An antifibrillogenic agent for inhibiting amyloidosis and/or for cytoprotection, which comprises a peptide of Formula I, an isomer thereof, a retro or a retro-inverso isomer thereof or a peptidomimetic thereof:

$$\mathrm{Xaa_1\text{-}Xaa_2\text{-}Xaa_3\text{-}Xaa_4} \qquad \mathrm{I}$$

wherein,

$\mathrm{Xaa_1}$ is absent or selected from the group consisting of Lys, Lys-Lys, $\mathrm{Xaa_5}$-Lys-, and Ala;

$\mathrm{Xaa_5}$ is absent or selected from the group consisting of His-Gln-, His-His-Gln-, Val-His-His-Gln-, Glu-Val-His-His-Gln-, Asp-Asp-Asp-, Lys-Val-Asp-Asp-Gln-Asp-, Gln-;

$\mathrm{Xaa_2}$ is absent or any amino acid;

$\mathrm{Xaa_3}$ is absent, Val or Phe;

$\mathrm{Xaa_4}$ is absent or selected from the group consisting of Phe, Phe-$\mathrm{NH_2}$, Phe-Phe, Phe-Phe-Ala, Phe-Phe-Ala-$\mathrm{NH_2}$, Phe-Phe-Ala-Gln, Phe-Phe-Ala-Gln-$\mathrm{NH_2}$, Val-Leu-Lys, Val-Leu-Lys-$\mathrm{NH_2}$;

wherein said peptide of formula I contains at least one Lys or Asp;

and wherein said peptide has at least one [D] amino acid residue,

with the proviso that Lys-Lys-Leu-Val-Phe-Phe-Ala is an all-[D] peptide; and with the proviso that when $\mathrm{Xaa_5}$ is Lys-Val-Asp-Asp-Gln-Asp- all of $\mathrm{Xaa_2}$, $\mathrm{Xaa_3}$, and $\mathrm{Xaa_4}$ are absent.

2. The antifibrillogenic agent of claim 1, wherein $\mathrm{Xaa_2}$ is a hydrophobic amino acid residue.

3. The antifibrillogenic agent of claim 1, wherein the peptide of formula I has at least two [D] amino acid residues.

4. The antifibrillogenic agent of claim 1, wherein the peptide of formula I has at least three [D] amino acid residues.

5. The antifibrillogenic agent of claim 1, wherein the peptide of formula I has one [L] amino acid residue.

6. The antifibrillogenic agent of claim 1, wherein the peptide of formula I is an all-[D] isomer peptide.

7. The antifibrillogenic agent of claim 1, 2, 3, 4, 5, or 6, wherein said peptide of Formula I is selected from the group consisting of:

Lys-Ile-Val-Phe-Phe-Ala	(SEQ ID NO:1);
Lys-Lys-Leu-Val-Phe-Phe-Ala	(SEQ ID NO:2);
Lys-Leu-Val-Phe-Phe-Ala	(SEQ ID NO:3);
Lys-Phe-Val-Phe-Phe-Ala	(SEQ ID NO:4);
Ala-Phe-Phe-Val-Leu-Lys	(SEQ ID NO:5);
Lys-Leu-Val-Phe	(SEQ ID NO:6);
Lys-Ala-Val-Phe-Phe-Ala	(SEQ ID NO:7);
Lys-Leu-Val-Phe-Phe	(SEQ ID NO:8);
Lys-Val-Val-Phe-Phe-Ala	(SEQ ID NO:9);
Lys-Ile-Val-Phe-Phe-Ala-NH_2	(SEQ ID NO:10);
Lys-Leu-Val-Phe-Phe-Ala-NH_2	(SEQ ID NO:11);
Lys-Phe-Val-Phe-Phe-Ala-NH_2	(SEQ ID NO:12);
Ala-Phe-Phe-Val-Leu-Lys-NH_2	(SEQ ID NO:13);
Lys-Leu-Val-Phe-NH_2	(SEQ ID NO:14);
Lys-Ala-Val-Phe-Phe-Ala-NH_2	(SEQ ID NO:15);
Lys-Leu-Val-Phe-Phe-NH_2	(SEQ ID NO:16);
Lys-Val-Val-Phe-Phe-Ala-NH_2	(SEQ ID NO:17);
Lys-Leu-Val-Phe-Phe-Ala-Gln	(SEQ ID NO:18);
Lys-Leu-Val-Phe-Phe-Ala-Gln-NH_2	(SEQ ID NO:19);
His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-NH_2	(SEQ ID NO:20);
Asp-Asp-Asp	(SEQ ID NO:21);

Lys-Val-Asp-Asp-Gln-Asp (SEQ ID NO:22);
His-His-Gln-Lys (SEQ ID NO:23);
and
Gln-Lys-Leu-Val-Phe-Phe-NH_2 (SEQ ID NO:24).

8. The antifibrillogenic agent of claim 1, wherein the peptide of formula I is a peptide as set forth in SEQ ID NO:2 or SEQ ID NO:3.

9. A labeled conjugate for *in vivo* imaging of amyloid deposits, which comprises a conjugate of formula II:

$$A\text{-}B\text{-}C \qquad \text{II}$$

wherein A is an amyloid plaque-targeting moiety selected from the group consisting of a peptide of Formula I as defined in claim 1, an isomer thereof, a retro or a retro-inverso isomer thereof and a peptidomimetic thereof,
wherein B is a linker portion allowing attachment of the amyloid plaque-targeting moiety to C; and
wherein C is a label that allows for said *in vivo* imaging.

10. The labeled conjugate of claim 9, wherein Xaa_2 in Formula I is a hydrophobic amino acid residue.

11. The labeled conjugate of claim 9, wherein the peptide of formula I has at least two [D] amino acid residues.

12. The labeled conjugate of claim 9, wherein the peptide of formula I has at least three [D] amino acid residues.

13. The labeled conjugate of claim 9, wherein the peptide of formula I has one [L] amino acid residue.

14. The labeled conjugate of claim 9, wherein the peptide of formula I is an all-[D] isomer peptide.

15. The labeled conjugate of claim 9, 10, 11, 12, 13 or 14, wherein said peptide of Formula I is selected from the group consisting of:
Lys-Ile-Val-Phe-Phe-Ala (SEQ ID NO:1);
Lys-Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:2);
Lys-Leu-Val-Phe-Phe-Ala (SEQ ID NO:3);
Lys-Phe-Val-Phe-Phe-Ala (SEQ ID NO:4);
Ala-Phe-Phe-Val-Leu-Lys (SEQ ID NO:5);
Lys-Leu-Val-Phe (SEQ ID NO:6);
Lys-Ala-Val-Phe-Phe-Ala (SEQ ID NO:7);
Lys-Leu-Val-Phe-Phe (SEQ ID NO:8);
Lys-Val-Val-Phe-Phe-Ala (SEQ ID NO:9);
Lys-Ile-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:10);
Lys-Leu-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:11);
Lys-Phe-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:12);
Ala-Phe-Phe-Val-Leu-Lys-NH_2 (SEQ ID NO:13);
Lys-Leu-Val-Phe-NH_2 (SEQ ID NO:14);
Lys-Ala-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:15);
Lys-Leu-Val-Phe-Phe-NH_2 (SEQ ID NO:16);
Lys-Val-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:17);
Lys-Leu-Val-Phe-Phe-Ala-Gln (SEQ ID NO:18);
Lys-Leu-Val-Phe-Phe-Ala-Gln-NH_2 (SEQ ID NO:19);
His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-NH_2 (SEQ ID NO:20);
Asp-Asp-Asp (SEQ ID NO:21);
Lys-Val-Asp-Asp-Gln-Asp (SEQ ID NO:22);
His-His-Gln-Lys (SEQ ID NO:23);
and
Gln-Lys-Leu-Val-Phe-Phe-NH2 (SEQ ID NO:24).

16. The labeled conjugate of claim 15, wherein B is selected from the group consisting of Glucose and Phe.

17. The labeled conjugate of claim 15, wherein C is ^{99m}Tc.

18. A method for the treatment of amyloidosis disorders in a patient, which comprises administering to said patient a therapeutically effective amount of a peptide of Formula I as defined in claim 1, 2, 3, 4, 5, 6, 7 or 8.

19. A method for the treatment of amyloidosis disorders in a patient, which comprises administering to said patient a therapeutically effective amount of an antifibrillogenic agent as defined in claim 1, 2, 3, 4, 5, 6, 7 or 8.

20. A composition for the treatment of amyloidosis disorders in a patient, which comprises a therapeutically effective amount of a peptide of Formula I as defined in claim 1, 2, 3, 4, 5, 6, 7 or 8 in association with a pharmaceutically acceptable carrier.

21. A composition for the treatment of amyloidosis disorders in a patient, which comprises a therapeutically effective amount of an antifibrillogenic agent as defined in claim 1, 2, 3, 4, 5, 6, 7 or 8 in association with a pharmaceutically acceptable carrier.

22. A composition for *in vivo* imaging of amyloid deposits, which comprises a therapeutically effective amount of a labeled conjugate as defined in claim 9,

10, 11, 12, 13, 14, 15, 16 or 17 in association with a pharmaceutically acceptable carrier.

23. Use of a peptide of Formula I as defined in claim 1, 2, 3, 4, 5, 6, 7 or 8 for inhibiting amyloidosis and/or for cytoprotection.

24. Use of an antifibrillogenic agent as defined in claim 1, 2, 3, 4, 5, 6, 7 or 8 for inhibiting amyloidosis and/or for cytoprotection.

25. Use of a labeled conjugate as defined in claim 10, 11, 12, 13, 14, 15, 16 or 17 for *in vivo* imaging of amyloid deposits.

26. Use of a peptide of Formula I as defined in claim 1, 2, 3, 4, 5, 6, 7 or 8 for the manufacture of a medicament for inhibiting amyloidosis and/or for cytoprotection.

27. Use of an antifibrillogenic agent as defined in claim 1, 2, 3, 4, 5, 6, 7 or 8 for the manufacture of a medicament for inhibiting amyloidosis and/or for cytoprotection.

28. Use of a labeled conjugate as defined in claim 10, 11, 12, 13, 14, 15, 16 or 17 for the manufacture of a medicament for *in vivo* imaging of amyloid deposits.

29. A peptide, an isomer thereof, a retro or a retro-inverso isomer thereof or a peptidomimetic thereof, for inhibiting amyloidosis and/or for cytoprotection, said peptide having a sequence taken from the β-sheet region of an amyloid protein.

30. Use of a peptide as defined in claim 29 for inhibiting amyloidosis and/or for cytoprotection.

31. Use of a peptide as defined in claim 29 for the manufacture of a medicament for inhibiting amyloidosis and/or for cytoprotection.

32. A composition for inhibiting amyloidosis and/or for cytoprotection, which comprises a therapeutically effective amount of a peptide as defined in claim 31, 30 or 31 in association with a pharmaceutically acceptable carrier.

33. Use of a labeled peptide as defined in claim 29 for the manufacture of a medicament for *in vivo* imaging of amyloid deposits.

34. A process for the preparation of cells suitable for transplantation into a mammal, which cells are capable of forming amyloid deposits, said process comprising contacting the cells *in vitro* with the peptide of Formula I as defined in claim 1 or with the antifibrillogenic compound as defined in claim 1, 2, 3, 4, 5, 6, 7 or 8 for inhibiting amyloid deposit formation.

35. Process according to claim 34, wherein said peptide of Formula I or said antifibrillogenic compound causes breakdown of amyloid deposits, the deposits having been formed by said cells prior to said contact.

36. Process according to claim 34 or 35, in which the cells are cultured in the presence of the peptide of Formula I or the antifibrillogenic compound.

Protein - Protein Interaction: Targetted Sites

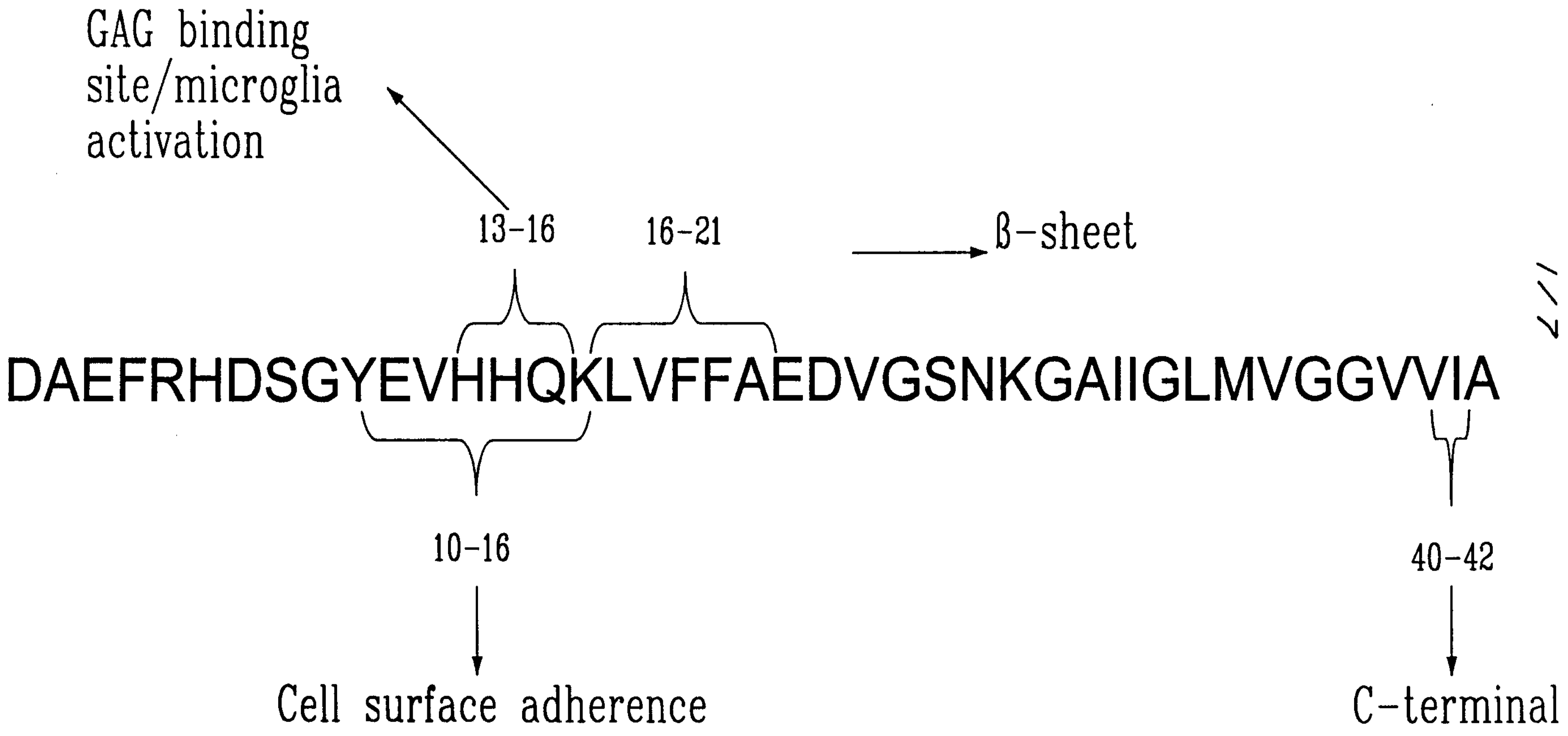


FIG-1

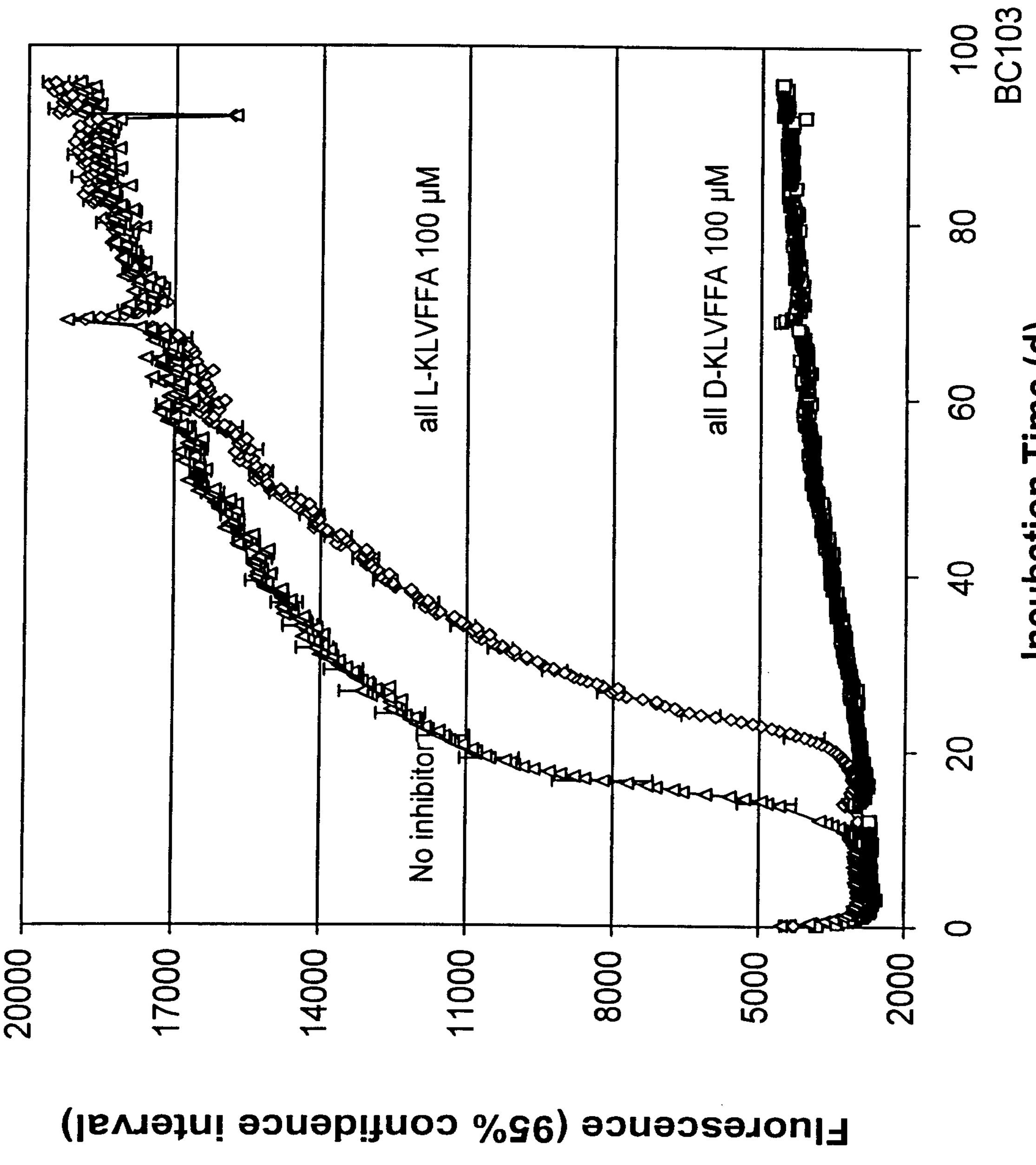
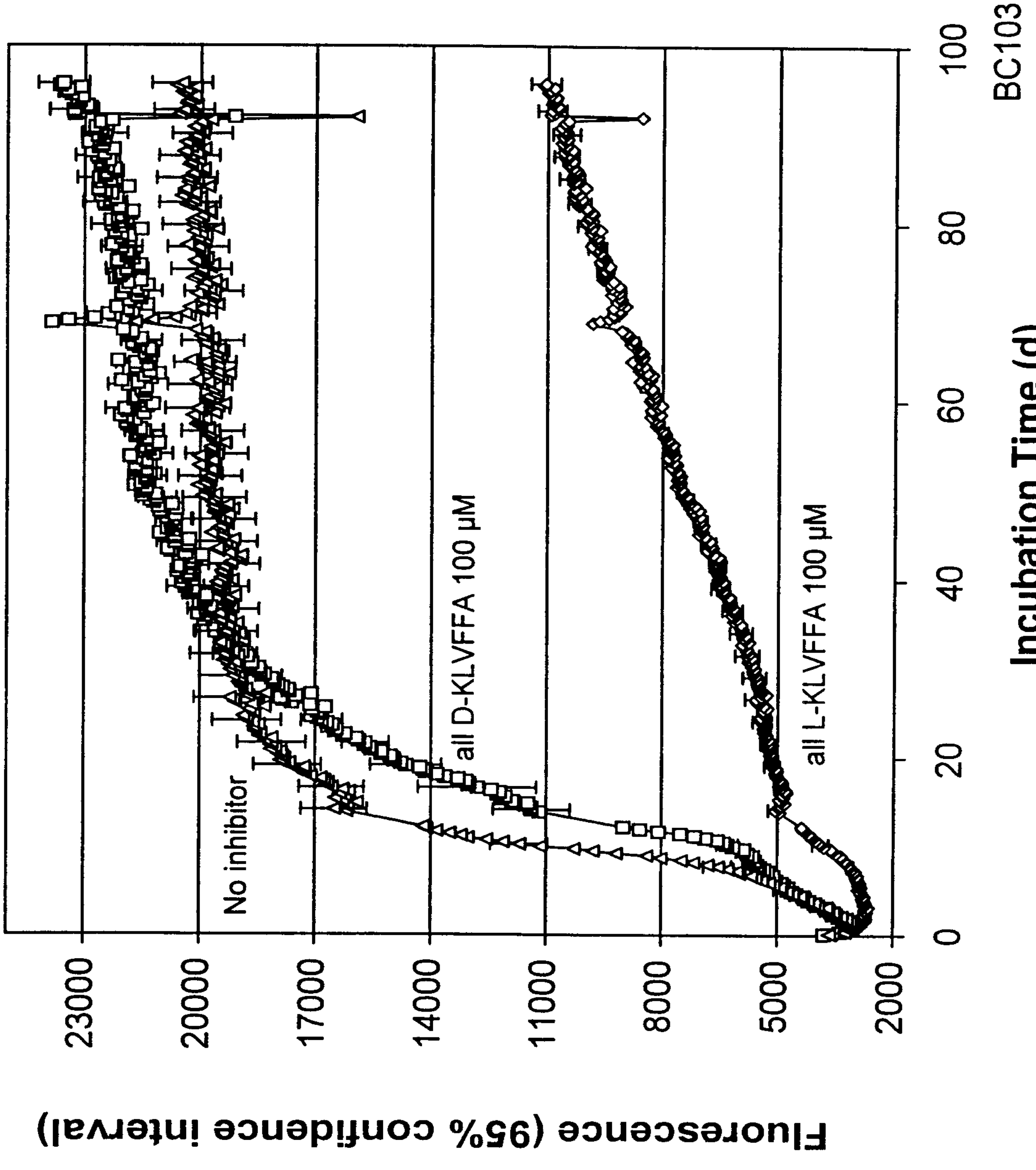

Fluorescence (95% confidence interval)
20000
17000
14000
11000
8000
5000
2000
0
20
40
60
80
100
Incubation Time (d)
No inhibitor
all L-KLVFFA 100 µM
all D-KLVFFA 100 µM
BC103

FIG. 2


Fluorescence (95% confidence interval)
23000
20000
17000
14000
11000
8000
5000
2000
0
20
40
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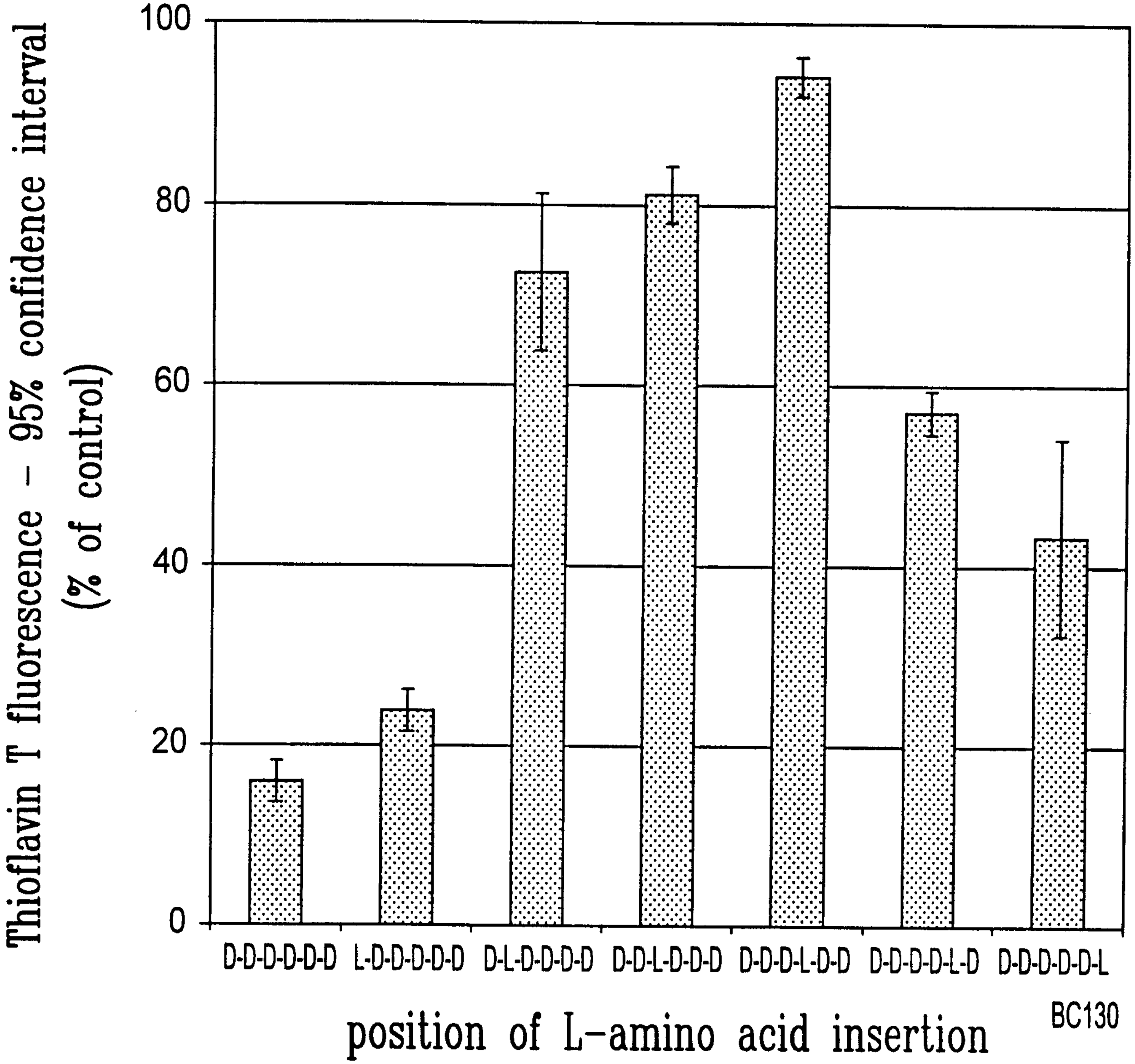
80
100
Incubation Time (d)
No inhibitor
all D-KLVFFA 100 µM
all L-KLVFFA 100 µM
BC103

FIG. 3


Thioflavin T fluorescence - 95% confidence interval
(% of control)
100
80
60
40
20
0
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D-L-D-D-D-D
D-D-L-D-D-D
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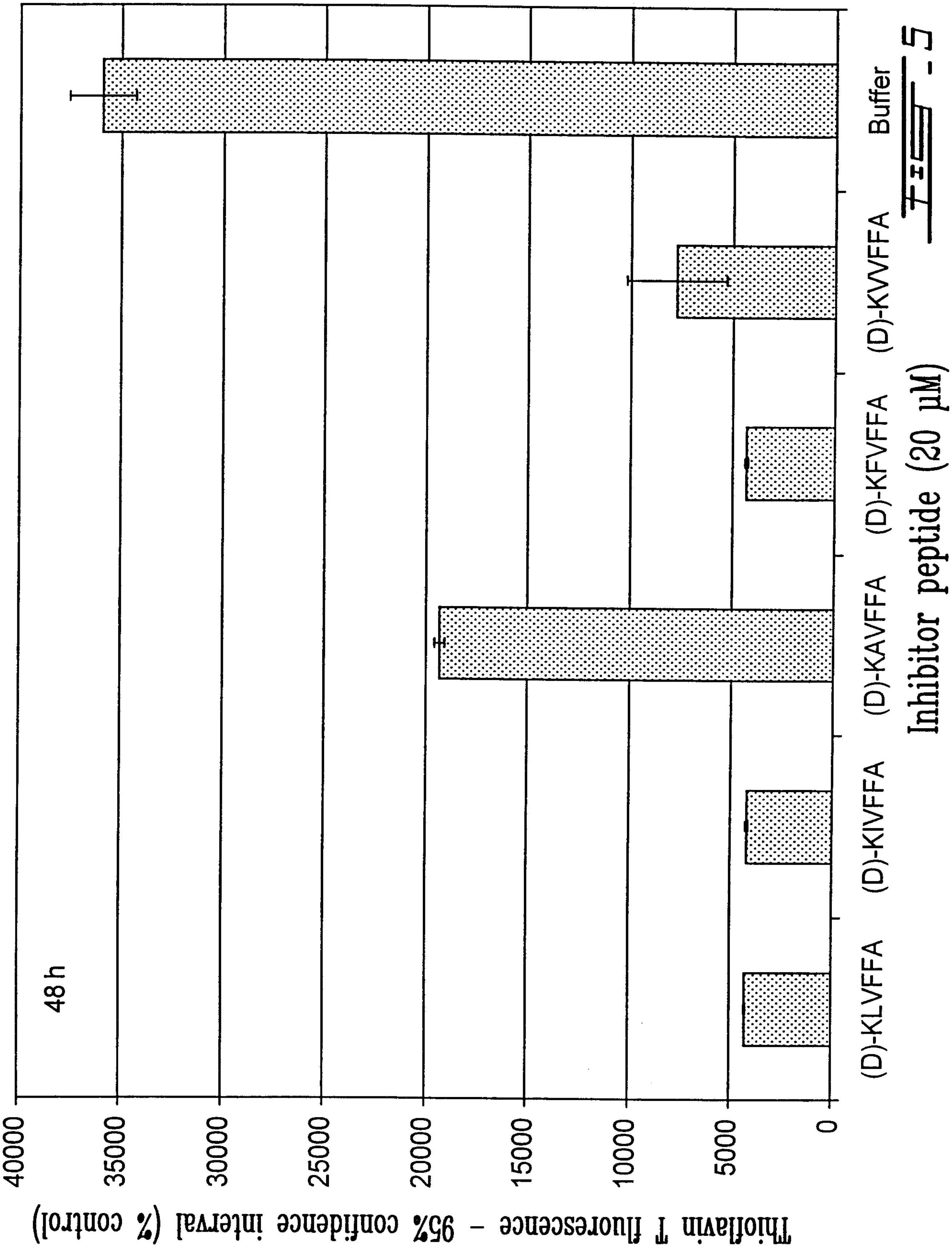
D-D-D-D-D-L
BC130
position of L-amino acid insertion

FIG. 4


48 h
40000
35000
30000
25000
20000
15000
10000
5000
0
Thioflavin T fluorescence - 95% confidence interval (% control)
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(D)-KIVFFA
(D)-KAVFFA
(D)-KFVFFA
(D)-KVVFFA
Buffer
Inhibitor peptide (20 µM)

Fig - 5

6/7

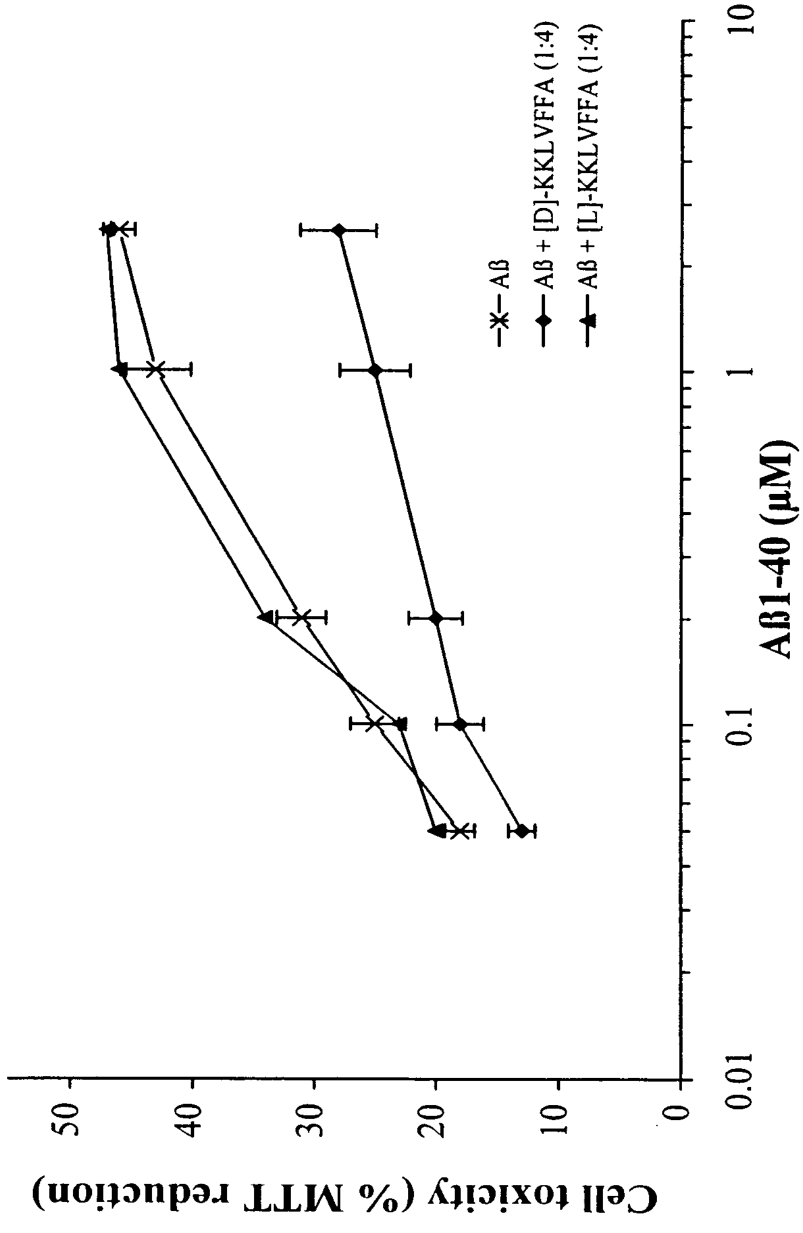
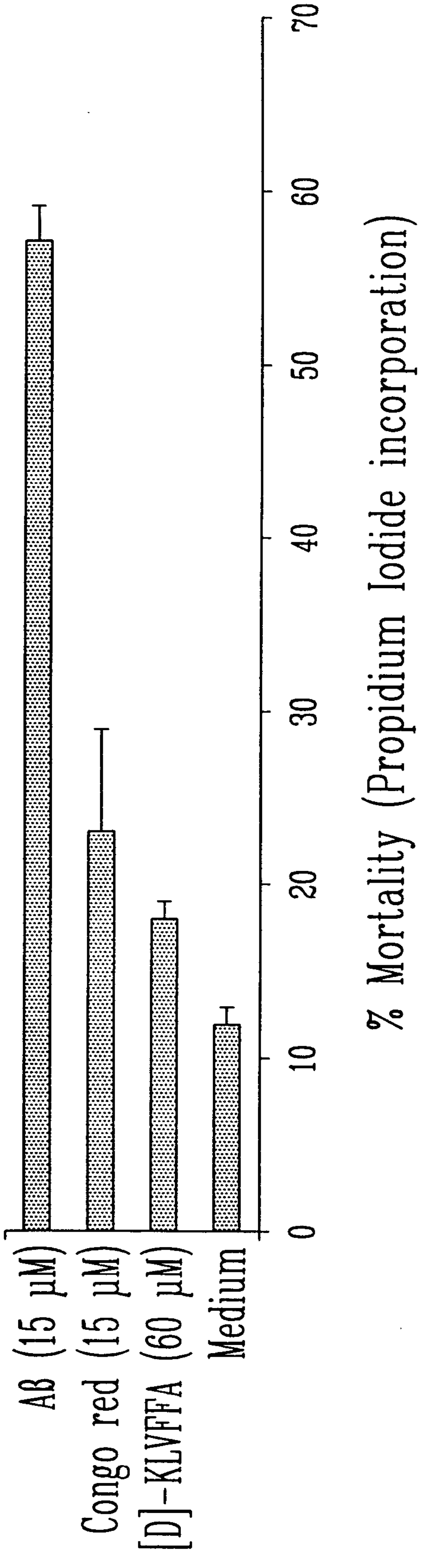

Cell toxicity (% MTT reduction)
0
10
20
30
40
50
0.01
0.1
1
10
Aß1-40 (µM)
—✱— Aß
—◆— Aß + [D]-KKLVFFA (1:4)
—▲— Aß + [L]-KKLVFFA (1:4)

FIG. 6



Aß (15 µM)
Congo red (15 µM)
[D]-KLVFFA (60 µM)
Medium
0
10
20
30
40
50
60
70
% Mortality (Propidium Iodide incorporation)

FIG. 7

```
                    SEQUENCE LISTING

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            CHALIFOUR, Robert
            GERVAIS, Francine
            GUPTA, Ajay

      <120> STEREOSELECTIVE ANTIFIBRILLOGENIC
        PEPTIDES AND PEPTIDOMIMETICS THEREOF


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             cytoprotection

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(54) **Title:** PREVENTION AND TREATMENT OF AMYLOIDOGENIC DISEASE

(57) **Abstract:** The invention provides improved agents and methods for treatment of diseases associated with amyloid deposits of Aβ in the brain of a patient. Such methods entail administering agents that induce a beneficial immunogenic response against the amyloid deposit. The methods are useful for prophylactic and therapeutic treatment of Alzheimer's disease. Preferred agents including N-terminal fragments of Aβ and antibodies binding to the same.

PREVENTION AND TREATMENT OF AMYLOIDOGENIC DISEASE

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is a continuation in part of USSN 09/322,289, filed May 28, 1999, which is incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

The invention resides in the technical fields of immunology and medicine.

BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is a progressive disease resulting in senile dementia. *See generally* Selkoe, *TINS* 16, 403-409 (1993); Hardy et al., WO 92/13069; Selkoe, *J. Neuropathol. Exp. Neurol.* 53, 438-447 (1994); Duff et al., *Nature* 373, 476-477 (1995); Games et al., *Nature* 373, 523 (1995). Broadly speaking, the disease falls into two categories: late onset, which occurs in old age (65 + years) and early onset, which develops well before the senile period, i.e., between 35 and 60 years. In both types of disease, the pathology is the same but the abnormalities tend to be more severe and widespread in cases beginning at an earlier age. The disease is characterized by at least two types of lesions in the brain, senile plaques and neurofibrillary tangles. Senile plaques are areas of disorganized neuropil up to 150 μm across with extracellular amyloid deposits at the center visible by microscopic analysis of sections of brain tissue. Neurofibrillary tangles are intracellular deposits of microtubule associated tau protein consisting of two filaments twisted about each other in pairs.

The principal constituent of the plaques is a peptide termed Aβ or β-amyloid peptide. Aβ peptide is an internal fragment of 39-43 amino acids of a precursor protein termed amyloid precursor protein (APP). Several mutations within the APP protein have been correlated with the presence of Alzheimer's disease. See, e.g., Goate et al., *Nature* 349, 704) (1991) (valine^{717} to isoleucine); Chartier Harlan et al. *Nature* 353, 844 (1991)) (valine^{717} to glycine); Murrell et al., *Science* 254, 97 (1991) (valine^{717} to phenylalanine); Mullan et al., *Nature Genet.* 1, 345 (1992) (a double mutation changing lysine^{595}-methionine^{596} to asparagine^{595}-leucine^{596}). Such mutations are thought to cause Alzheimer's disease by increased or altered processing of APP to Aβ, particularly processing of APP to increased amounts of the long form of Aβ (i.e., Aβ1-42 and Aβ1-43). Mutations in other genes, such as the presenilin genes, PS1 and PS2, are thought indirectly to affect processing of APP to generate increased amounts of long form Aβ (see Hardy, *TINS* 20, 154 (1997)). These observations indicate that Aβ, and particularly its long form, is a causative element in Alzheimer's disease.

McMichael, EP 526,511, proposes administration of homeopathic dosages (less than or equal to 10^{-2} mg/day) of Aβ to patients with preestablished AD. In a typical human with about 5 liters of plasma, even the upper limit of this dosage would be expected to generate a concentration of no more than 2 pg/ml. The normal concentration of Aβ in human plasma is typically in the range of 50-200 pg/ml (Seubert et al., *Nature* 359, 325-327 (1992)). Because EP 526,511's proposed dosage would barely alter the level of endogenous circulating Aβ and because EP 526,511 does not recommend use of an adjuvant, as an immunostimulant, it seems implausible that any therapeutic benefit would result.

By contrast, the present invention is directed *inter alia* to treatment of Alzheimer's and other amyloidogenic diseases by administration of fragments of Aβ , or antibody to certain epitopes within Aβ to a patient under conditions that generate a beneficial immune response in the patient. The invention thus fulfills a longstanding need for therapeutic regimes for preventing or ameliorating the neuropathology and, in some patients, the cognitive impairment associated with Alzheimer's disease.

This application is related to WO99/27944, filed November 30, 1998, USSN 60/067,740, filed December 2, 1997, USSN 60/080,970, filed April 7, 1998, and USSN 09/201,430, filed November 30, 1998, each of which is incorporated by reference in its entirety for all purposes.

SUMMARY OF THE CLAIMED INVENTION

In one aspect, the invention provides methods of preventing or treating a disease associated with amyloid deposits of Aβ in the brain of a patient. Such diseases include Alzheimer's disease, Down's syndrome and cognitive impairment. The latter can occur with or without other characteristics of an amyloidogenic disease. Some methods of the invention entail administering an effective dosage of an antibody that specifically binds to a component of an amyloid deposit to the patient. Such methods are particularly useful for preventing or treating Alzheimer's disease in human patients. Some methods entail administering an effective dosage of an antibody that binds to Aβ. Some methods entail administering an effective dosage of an antibody that specifically binds to an epitope within residues 1-10 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 1-6 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 1-5 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 1-7 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 3-7 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 1-3 of Aβ. In some methods, the antibody specifically binds to an epitope within residues 1-4 of Aβ. In some methods, the antibody binds to an epitope comprising a free N-terminal residue of Aβ. In some methods, the antibody binds to an epitope within residues of 1-10 of Aß wherein residue 1 and/or residue 7 of Aβ is aspartic acid. In some methods, the antibody specifically binds to Aβ peptide without binding to full-length amyloid precursor protein (APP). In some methods, the isotype of the antibody is human IgG1.

In some methods, the antibody binds to an amyloid deposit in the patient and induces a clearing response against the amyloid deposit. For example, such a clearing response can be effected by Fc receptor mediated phagocytosis.

The methods can be used on both asymptomatic patients and those currently showing symptoms of disease. The antibody used in such methods can be a human, humanized, chimeric or nonhuman antibody and can be monoclonal or polyclonal. In some methods, the antibody is prepared from a human immunized with Aβ peptide, which human can be the patient to be treated with antibody.

In some methods, the antibody is administered with a pharmaceutical carrier as a pharmaceutical composition. In some methods, antibody is administered at a dosage of 0.0001 to 100 mg/kg, preferably, at least 1 mg/kg body weight antibody. In some methods, the antibody is administered in multiple dosages over a prolonged period, for example, of at least six months. In some methods, the antibody is administered as a sustained release composition. The antibody can be administered, for example, intraperitoneally, orally, subcutaneously, intracranially, intramuscularly, topically, intranasally or intravenously.

In some methods, the antibody is administered by administering a polynucleotide encoding at least one antibody chain to the patient. The polynucleotide is expressed to produce the antibody chain in the patient. Optionally, the polynucleotide encodes heavy and light chains of the antibody. The polynucleotide is expressed to produce the heavy and light chains in the patient. In some methods, the patient is monitored for level of administered antibody in the blood of the patient.

In another aspect, the invention provides methods of preventing or treating a disease associated with amyloid deposits of Aβ in the brain of patient. For example, the methods can be used to treat Alzheimer's disease or Down's syndrome or cognitive impairment. Such methods entail administering fragments of Aβ or analogs thereof eliciting an immunogenic response against certain epitopes within Aβ. Some methods entail administering to a patient an effective dosage of a polypeptide comprising an N-terminal segment of at least residues 1-5 of Aβ, the first residue of Aβ being the N-terminal residue of the polypeptide, wherein the polypeptide is free of a C-terminal segment of Aβ. Some methods entail administering to a patient an effective dosage of a polypeptide comprising an N-terminal segment of Aβ, the segment beginning at residue 1-3 of Aβ and ending at residues 7-11 of Aβ. Some methods entail administering to a patient an effective dosage of an agent that induces an immunogenic response against an N-terminal segment of Aβ, the segment beginning at residue 1-3 of Aβ and ending at residues 7-11 of Aβ without inducing an immunogenic response against an epitope within residues 12-43 of Aβ43.

In some of the above methods, the N-terminal segment of Aβ is linked at its C-terminus to a heterologous polypeptide. In some of the above methods, the N-terminal segment of Aβ is linked at its N-terminus to a heterologous polypeptide. In some of the above methods, the N-terminal segment of Aβ is linked at its N and C termini to first and second heterologous polypeptides. In some of the above methods, the N-terminal segment of Aβ is linked at its N terminus to a heterologous polypeptide, and at its C-terminus to at least one additional copy of the N-terminal segment. In some of the above methods, the heterologous polypeptide and thereby a B-cell response against the N-terminal segment. In some of the above methods, the polypeptide further comprises at least one additional copy of the N-terminal segment. In some of the above methods, the polypeptide comprises from N-terminus to C-terminus, the N-terminal segment of Aβ, a plurality of additional copies of the N-terminal segment, and the heterologous amino acid segment. In some of the above methods, the N-terminal segment consists of Aβ1-7. In some of the above methods, the N-terminal segment consists of Aβ3-7.

In some methods, the fragment is free of at least the 5 C-terminal amino acids in Aβ43. In some methods, the fragment comprises up to 10 contiguous amino acids from Aβ. Fragments are typically administered at greater than 10 micrograms per dose per patient.

In some methods, the fragment is administered with an adjuvant that enhances the immune response to the Aβ peptide. The adjuvant and fragment can be administered in either order or together as a composition. The adjuvant can be, for example, aluminum hydroxide, aluminum phosphate, MPL^{TM}, QS-21 (Stimulon^{TM}) or incomplete Freund's adjuvant.

The invention further provides pharmaceutical compositions comprising fragments of Aβ or other agents eliciting immunogenic response to the same epitopes of Aβ, such as described above, and an adjuvant. The invention also provides pharmaceutical compositions comprising any of the antibodies described above and a pharmaceutically acceptable carrier.

In another aspect, the invention provides methods of screening an antibody for activity in treating a disease associated with deposits of Aβ in the brain of a patient (e.g., Alzheimer's disease). Such methods entail contacting the antibody with a polypeptide comprising at least five contiguous amino acids of an N-terminal segment of Aβ beginning at a residue between 1 and 3 of Aβ, the polypeptide being free of a C-terminal segment of Aβ. One then determines whether the antibody specifically binds to the polypeptide, specific binding providing an indication that the antibody has activity in treating the disease.

In another aspect, the invention provides methods of screening an antibody for activity in clearing an antigen-associated biological entity. Such methods entail combining the antigen-associated biological entity and the antibody and phagocytic cells bearing Fc receptors in a medium. The amount of the antigen-associated biological entity remaining in the medium is then monitored. A reduction in the amount of the antigen-associated biological entity indicates the antibody has clearing activity against the antigen-associated biological entity. The antigen can be provided as a tissue sample or in isolated form. For example, the antigen can be provided as a tissue sample from the brain of an Alzheimer's disease patient or a mammal animal having Alzheimer's pathology.

Other tissue samples against which antibodies can be tested for clearing activity include cancerous tissue samples, virally infected tissue samples, tissue samples comprising inflammatory cells, nonmalignant abnormal cell growths, or tissue samples comprising an abnormal extracellular matrix.

In another aspect, the invention provides methods of detecting an amyloid deposit in a patient. Such methods entail administering to the patient an antibody that specifically binds to an epitope within amino acids 1-10 of Aβ, and detecting presence of the antibody in the brain of the patient. In some methods, the antibody binds to an epitope within residues 4-10 of Aβ. In some methods, the antibody is labelled with a paramagnetic label and detected by nuclear magnetic resonance tomography.

The invention further provides diagnostic kits suitable for use in the above methods. Such a kit comprises an antibody that specifically binds to an epitope with residues 1-10 of Aβ. Some kits bear a label describing use of the antibody for in vivo diagnosis or monitoring of Alzheimer's disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Antibody titer after injection of transgenic mice with Aβ1-42.

Fig. 2: Amyloid burden in the hippocampus. The percentage of the area of the hippocampal region occupied by amyloid plaques, defined by reactivity with the Aβ-specific monoclonal antibody 3D6, was determined by computer-assisted quantitative image analysis of immunoreacted brain sections. The values for individual mice are shown sorted by treatment group. The horizontal line for each grouping indicates the median value of the distribution.

Fig 3: Neuritic dystrophy in the hippocampus. The percentage of the area of the hippocampal region occupied by dystrophic neurites, defined by their reactivity with the human APP-specific monoclonal 8E5, was determined by quantitative computer-assisted image analysis of immunoreacted brain sections. The values for individual mice are shown for the AN1792-treated group and the PBS-treated control group. The horizontal line for each grouping indicates the median value of the distribution.

Fig. 4: Astrocytosis in the retrosplenial cortex. The percentage of the area of the cortical region occupied by glial fibrillary acidic protein (GFAP)-positive astrocytes was determined by quantitative computer-assisted image analysis of

immunoreacted brain sections. The values for individual mice are shown sorted by treatment group and median group values are indicated by horizontal lines.

Fig. 5: Geometric mean antibody titers to Aβ1-42 following immunization with a range of eight doses of AN1792 containing 0.14, 0.4, 1.2, 3.7, 11, 33, 100, or 300 μg.

Fig. 6: Kinetics of antibody response to AN1792 immunization. Titers are expressed as geometric means of values for the 6 animals in each group.

Fig. 7: Quantitative image analysis of the cortical amyloid burden in PBS- and AN1792-treated mice.

Fig. 8: Quantitative image analysis of the neuritic plaque burden in PBS- and AN1792-treated mice.

Fig. 9: Quantitative image analysis of the percent of the retrosplenial cortex occupied by astrocytosis in PBS- and AN1792-treated mice.

Fig. 10: Lymphocyte Proliferation Assay on spleen cells from AN1792-treated (upper panel) or PBS-treated (lower panel).

Fig. 11: Total Aβ levels in the cortex. A scatterplot of individual Aβ profiles in mice immunized with Aβ or APP derivatives combined with Freund' adjuvant.

Fig. 12: Amyloid burden in the cortex was determined by quantitative image analysis of immunoreacted brain sections for mice immunized with the Aβ peptide conjugates Aβ1-5, Aβ1-12, and Aβ13-28; the full length Aβ aggregates AN1792 (Aβ1-42) and AN1528 (Aβ1-40) and the PBS-treated control group.

Fig. 13: Geometric mean titers of Aβ-specific antibody for groups of mice immunized with Aβ or APP derivatives combined with Freund's adjuvant.

Fig. 14: Geometric mean titers of Aβ-specific antibody for groups of guinea pigs immunized with AN1792, or a palmitoylated derivative thereof, combined with various adjuvants.

Fig: 15(A-E): Aβ levels in the cortex of 12-month old PDAPP mice treated with AN1792 or AN1528 with different adjuvants.

Fig. 16: Mean titer of mice treated with polyclonal antibody to Aβ.

Fig. 17: Mean titer of mice treated with monoclonal antibody 10D5 to Aβ.

Fig. 18: Mean titer of mice treated with monoclonal antibody 2F12 to Aβ.

Fig. 19: Epitope Map: Restricted N–terminal Response. Day 175 serum from cynomolgus monkeys was tested by ELISA against a series of 10–mer overlapping

peptides covering the complete AN1792 sequence. Animal number F10920M shows a representative N–terminal restricted response to the peptide DAEFRHDSGY which covers amino acids 1–10 of the AN1792 peptide which was used as immunizing antigen.

Fig. 20: Epitope Map: Non–restricted N–terminal response. Day 175 serum from cynomolgus monkeys was tested by ELISA against a series of 10-mer overlapping peptides covering the complete AN1792 sequence. Animal number F10975F shows a representative non–restricted N–terminal response. Reactivity is seen against the two peptides N–terminal and one peptide C-terminal to the peptide DAEFRHDSGY which covers amino acids 1–10 of the AN1792 peptide.

DEFINITIONS

The term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 65 percent sequence identity, preferably at least 80 or 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (*e.g.*, 99 percent sequence identity or higher). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally* Ausubel *et al., supra*). One example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al., J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Typically, default program parameters can be used to perform the sequence comparison, although customized parameters can also be used. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89, 10915 (1989))

For purposes of classifying amino acids substitutions as conservative or nonconservative, amino acids are grouped as follows: Group I (hydrophobic sidechains): norleucine, met, ala, val, leu, ile; Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Conservative substitutions involve substitutions between amino acids in the same class. Non-conservative substitutions constitute exchanging a member of one of these classes for a member of another.

Therapeutic agents of the invention are typically substantially pure from undesired contaminant. This means that an agent is typically at least about 50% w/w (weight/weight) purity, as well as being substantially free from interfering proteins and contaminants. Sometimes the agents are at least about 80% w/w and, more preferably at least 90 or about 95% w/w purity. However, using conventional protein purification techniques, homogeneous peptides of at least 99% w/w can be obtained.

Specific binding between two entities means an affinity of at least 10^6, 10^7, $10^{8,}$ 10^9 M^{-1}, or 10^{10} M^{-1}. Affinities greater than 10^8 M^{-1} are preferred.

The term "antibody" or “immunoglobulin” is used to include intact antibodies and binding fragments thereof. Typically, fragments compete with the intact antibody from which they were derived for specific binding to an antigen fragment including separate heavy chains, light chains Fab, Fab' F(ab')2, Fabc, and Fv. Fragments are produced by recombinant DNA techniques, or by enzymatic or chemical separation of intact immunoglobulins. The term “antibody” also includes one or more immunoglobulin chains that are chemically conjugated to, or expressed as, fusion proteins with other proteins. The term “antibody” also includes bispecific antibody. A bispecific or bifunctional antibody is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, e.g., Songsivilai & Lachmann, *Clin. Exp. Immunol.* 79:315-321 (1990); Kostelny et al., *J. Immunol.* 148, 1547-1553 (1992).

APP^{695}, APP^{751}, and APP^{770} refer, respectively, to the 695, 751, and 770 amino acid residue long polypeptides encoded by the human APP gene. See Kang et al., *Nature* 325, 773 (1987); Ponte et al., *Nature* 331, 525 (1988); and Kitaguchi et al., *Nature* 331, 530 (1988). Amino acids within the human amyloid precursor protein (APP) are assigned numbers according to the sequence of the APP770 isoform. Terms such as Aβ39, Aβ40, Aβ41, Aβ42 and Aβ43 refer to an Aβ peptide containing amino acid residues 1-39, 1-40, 1-41, 1-42 and 1-43.

An “antigen” is an entity to which an antibody specifically binds.

The term "epitope" or "antigenic determinant" refers to a site on an antigen to which B and/or T cells respond. B-cell epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols in Methods in Molecular Biology,* Vol. 66, Glenn E. Morris, Ed. (1996). Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen. T-cells recognize continuous epitopes of about nine amino acids for CD8 cells or about 13-15 amino acids for CD4 cells. T cells that recognize the epitope can be identified by *in vitro* assays that measure antigen-dependent proliferation, as determined by ^{3}H-thymidine incorporation by primed T cells in response to an epitope (Burke et al., *J. Inf. Dis.* 170, 1110-19 (1994)), by antigen-dependent killing (cytotoxic T lymphocyte assay, Tigges et al., *J. Immunol.* 156, 3901-3910) or by cytokine secretion.

The term "immunological" or "immune" response is the development of a beneficial humoral (antibody mediated) and/or a cellular (mediated by antigen-specific T cells or their secretion products) response directed against an amyloid peptide in a recipient patient. Such a response can be an active response induced by administration of immunogen or a passive response induced by administration of antibody or primed T-cells. A cellular immune response is elicited by the presentation of polypeptide epitopes in association with Class I or Class II MHC molecules to activate antigen-specific $CD4^+$ T helper cells and/or $CD8^+$ cytotoxic T cells. The response may also involve activation of monocytes, macrophages, NK cells, basophils, dendritic cells, astrocytes, microglia cells, eosinophils or other components of innate immunity. The presence of a cell-mediated immunological response can be determined by proliferation assays ($CD4^+$ T cells) or CTL (cytotoxic T lymphocyte) assays (see Burke, *supra*; Tigges, *supra*). The relative contributions of humoral and cellular responses to the protective or therapeutic effect of an immunogen can be distinguished by separately isolating antibodies and T-cells from an immunized syngeneic animal and measuring protective or therapeutic effect in a second subject.

An "immunogenic agent" or "immunogen" is capable of inducing an immunological response against itself on administration to a mammal, optionally in conjunction with an adjuvant.

The term "naked polynucleotide" refers to a polynucleotide not complexed with colloidal materials. Naked polynucleotides are sometimes cloned in a plasmid vector.

The term "adjuvant" refers to a compound that when administered in conjunction with an antigen augments the immune response to the antigen, but when administered alone does not generate an immune response to the antigen. Adjuvants can augment an immune response by several mechanisms including lymphocyte recruitment, stimulation of B and/or T cells, and stimulation of macrophages.

The term "patient" includes human and other mammalian subjects that receive either prophylactic or therapeutic treatment.

Disaggregated or monomeric Aβ means soluble, monomeric peptide units of Aβ. One method to prepare monomeric Aβ is to dissolve lyophilized peptide in neat DMSO with sonication. The resulting solution is centrifuged to remove any insoluble particulates. Aggregated Aβ is a mixture of oligomers in which the monomeric units are held together by noncovalent bonds.

Competition between antibodies is determined by an assay in which the immunoglobulin under test inhibits specific binding of a reference antibody to a common antigen, such as Aβ. Numerous types of competitive binding assays are known, for example: solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay (see Stahli et al., Methods in Enzymology 9:242-253 (1983)); solid phase direct biotin-avidin EIA (see Kirkland et al., J. Immunol. 137:3614-3619 (1986)); solid phase direct labeled assay, solid phase direct labeled sandwich assay (see Harlow and Lane, "Antibodies, A Laboratory Manual," Cold Spring Harbor Press (1988)); solid phase direct label RIA using I-125 label (see Morel et al., Molec. Immunol. 25(1):7-15 (1988)); solid phase direct biotin-avidin EIA (Cheung et al., Virology 176:546-552 (1990)); and direct labeled RIA (Moldenhauer et al., Scand. J. Immunol. 32:77-82 (1990)). Typically, such an assay involves the use of purified antigen bound to a solid surface or cells bearing either of these, an unlabelled test immunoglobulin and a labelled reference immunoglobulin. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test immunoglobulin. Usually the test immunoglobulin is present in excess. Antibodies identified by competition assay (competing antibodies) include antibodies binding to the same epitope as the reference antibody and antibodies binding to an adjacent epitope sufficiently proximal to the epitope bound by the reference antibody for steric hindrance to occur. Usually, when a competing antibody is present in excess, it will inhibit specific binding of a reference antibody to a common antigen by at least 50 or 75%.

Compositions or methods "comprising" one or more recited elements may include other elements not specifically recited. For example, a composition that comprises Aβ peptide encompasses both an isolated Aβ peptide and Aβ peptide as a component of a larger polypeptide sequence.

DETAILED DESCRIPTION

I. General

Several amyloidogenic diseases and conditions are characterized by presence of deposits of Aβ peptide aggregated to an insoluble mass in the brain of a patient. Such diseases include Alzheimer's disease, Down's syndrome and cognitive impairment. The latter is a symptom of Alzheimer's disease and Down's syndrome but can also without other characteristics of either of these diseases. For example, mild cognitive impairment or age-associated memory loss occurs in some patient who have not yet developed, or may never develop full Alzheimer's disease. Mild cognitive impairment can be defined by score on the Mini-Mental State Exam in accordance with convention. Such diseases are characterized by aggregates of Aβ that have a β-pleated sheet structure and stain with Congo Red dye. The basic approach of preventing or treating Alzheimer's disease or other amyloidogenic diseases by generating an immunogenic response to a component of the amyloid deposit in a patient is described in WO 99/27944 (incorporated by reference). The present application reiterates and confirms the efficacy of the basic approach. The present application is, however, principally directed to improved reagents and methods. These improvements are premised, in part, on the present inventors having localized the preferred epitopes within Aβ against which an immunogenic response should be directed. The identification of preferred epitopes within Aβ results in agents and methods having increased efficacy, reduced potential for side effects, and/or greater ease of manufacture, formulation and administration.

II. Therapeutic Agents

An immunogenic response can be active, as when an immunogen is administered to induce antibodies reactive with Aβ in a patient, or passive, as when an antibody is administered that itself binds to Aβ in a patient.

1. Agents Inducing Active Immune Response

Therapeutic agents induce an immunogenic response specifically directed to certain epitopes within Aβ peptides. Preferred agents are the Aβ peptide itself and segments thereof. Variants of such segments, analogs and mimetics of natural Aβ peptide that induce and/or crossreact with antibodies to the preferred epitopes of Aβ peptide can also be used.

Aβ, also known as β-amyloid peptide, or A4 peptide (see US 4,666,829; Glenner & Wong, Biochem. Biophys. Res. Commun. 120, 1131 (1984)), is a peptide of 39-43 amino acids, which is the principal component of characteristic plaques of Alzheimer's disease. Aβ is generated by processing of a larger protein APP by two enzymes, termed β and γ secretases (see Hardy, TINS 20, 154 (1997)). Known mutations in APP associated with Alzheimer's disease occur proximate to the site of β or γ secretase, or within Aβ. For example, position 717 is proximate to the site of γ-secretase cleavage of APP in its processing to Aβ, and positions 670/671 are proximate to the site of β-secretase cleavage. It is believed that the mutations cause AD by interacting with the cleavage reactions by which Aβ is formed so as to increase the amount of the 42/43 amino acid form of Aβ generated.

Aβ has the unusual property that it can fix and activate both classical and alternate complement cascades. In particular, it binds to Clq and ultimately to C3bi. This association facilitates binding to macrophages leading to activation of B cells. In addition, C3bi breaks down further and then binds to CR2 on B cells in a T cell dependent manner leading to a 10,000 increase in activation of these cells. This mechanism causes Aβ to generate an immune response in excess of that of other antigens.

Aβ has several natural occurring forms. The human forms of Aβ are referred to as Aβ39, Aβ40, Aβ41, Aβ42 and Aβ43. The sequences of these peptides and their relationship to the APP precursor are illustrated by Fig. 1 of Hardy et al., TINS 20, 155-158 (1997). For example, Aβ42 has the sequence:

H2N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala-OH.

Aβ41, Aβ40 and Aβ39 differ from Aβ42 by the omission of Ala, Ala-Ile, and Ala-Ile-Val respectively from the C-terminal end. Aβ43 differs from Aβ42 by the presence of a threonine residue at the C-terminus.

Immunogenic fragments of Aβ are advantageous relative to the intact molecule in the present methods for several reasons. First, because only certain epitopes within Aβ induce a useful immunogenic response for treatment of Alzheimer's disease, an equal dosage of mass of a fragment containing such epitopes provides a greater molar concentration of the useful immunogenic epitopes than a dosage of intact Aβ. Second, certain immunogenic fragments of Aβ generate an immunogenic response against amyloid deposits without generating a significant immunogenic response against APP protein from which Aβ derives. Third, fragments of Aβ are simpler to manufacture than intact Aβ due to their shorter size. Fourth, fragments of Aβ do not aggregate in the same manner as intact Aβ, simplifying preparation of pharmaceutical compositions and administration thereof.

Some immunogenic fragments of Aβ have a sequence of at least 2, 3, 5, 6, 10 or 20 contiguous amino acids from a natural peptide. Some immunogenic fragments have no more than 10, 9, 8, 7, 5 or 3 contiguous residues from Aβ. Fragments from the

N-terminal half of Aβ are preferred. Preferred immunogenic fragments include Aβ1-5, 1-6, 1-7, 1-10, 3-7, 1-3, and 1-4. The designation Aβ1-5 for example, indicates a fragment including residues 1-5 of Aβ and lacking other residues of Aβ. Fragments beginning at residues 1-3 of Aβ and ending at residues 7-11 of Aβ are particularly preferred. The fragment Aβ1-12 can also be used but is less preferred. In some methods, the fragment is an N-terminal fragment other than Aβ1-10. Other less preferred fragments include Aβ13-28, 17-28, 1-28, 25-35, 35-40 and 35-42. These fragments require screening for activity in clearing or preventing amyloid deposits as described in the Examples before use. Fragments lacking at least one, and sometimes at least 5 or 10 C-terminal amino acid present in a naturally occurring forms of Aβ are used in some methods. For example, a fragment lacking 5 amino acids from the C-terminal end of Aβ43 includes the first 38 amino acids from the N-terminal end of Aβ. Other components of amyloid plaques, for example, synuclein, and epitopic fragments thereof can also be used to induce an immunogenic response.

Unless otherwise indicated, reference to Aβ includes the natural human amino acid sequences indicated above as well as analogs including allelic, species and induced variants. Analogs typically differ from naturally occurring peptides at one, two or a few positions, often by virtue of conservative substitutions. Analogs typically exhibit at least 80 or 90% sequence identity with natural peptides. Some analogs also include unnatural amino acids or modifications of N or C terminal amino acids at a one, two or a few positions. For example, the natural aspartic acid residue at position 1 and/or 7 of Aβ can be replaced with iso-aspartic acid. Examples of unnatural amino acids are D-amino acids, α, α-disubstituted amino acids, N-alkyl amino acids, lactic acid, 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and isoaspartic acid. Fragments and analogs can be screened for prophylactic or therapeutic efficacy in transgenic animal models in comparison with untreated or placebo controls as described below.

Aβ, its fragments, and analogs can be synthesized by solid phase peptide synthesis or recombinant expression, or can be obtained from natural sources. Automatic peptide synthesizers are commercially available from numerous suppliers, such as Applied Biosystems, Foster City, California. Recombinant expression can be in bacteria, such as E. coli, yeast, insect cells or mammalian cells. Procedures for recombinant expression are described by Sambrook et al., *Molecular Cloning: A Laboratory Manual* (C.S.H.P. Press, NY 2d ed., 1989). Some forms of Aβ peptide are also available commercially (e.g., American Peptides Company, Inc., Sunnyvale, CA and California Peptide Research, Inc. Napa, CA).

Therapeutic agents also include longer polypeptides that include, for example, an active fragment of Aβ peptide, together with other amino acids. For example, preferred agents include fusion proteins comprising a segment of Aβ fused to a heterologous amino acid sequence that induces a helper T-cell response against the heterologous amino acid sequence and thereby a B-cell response against the Aβ segment. Such polypeptides can be screened for prophylactic or therapeutic efficacy in animal models in comparison with untreated or placebo controls as described below. The Aβ peptide, analog, active fragment or other polypeptide can be administered in associated or multimeric form or in dissociated form Therapeutic agents also include multimers of monomeric immunogenic agents.

In a further variation, an immunogenic peptide, such as a fragment of Aβ, can be presented by a virus or a bacteria as part of an immunogenic composition. A nucleic acid encoding the immunogenic peptide is incorporated into a genome or episome of the virus or bacteria. Optionally, the nucleic acid is incorporated in such a manner that the immunogenic peptide is expressed as a secreted protein or as a fusion protein with an outer surface protein of a virus or a transmembrane protein of a bacteria so that the peptide is displayed. Viruses or bacteria used in such methods should be nonpathogenic or attenuated. Suitable viruses include adenovirus, HSV, Venezuelan equine encephalitis virus and other alpha viruses, vesicular stomatitis virus, and other rhabdo viruses, vaccinia and fowl pox. Suitable bacteria include Salmonella and Shigella. Fusion of an immunogenic peptide to HBsAg of HBV is particularly suitable. Therapeutic agents also include peptides and other compounds that do not necessarily have a significant amino acid sequence similarity with Aβ but nevertheless serve as mimetics of Aβ and induce a similar immune response. For example, any peptides and proteins forming β-pleated sheets can be screened for suitability. Anti-idiotypic antibodies against monoclonal antibodies to Aβ or other amyloidogenic peptides can also be used. Such anti-Id antibodies mimic the antigen and generate an immune response to it (see *Essential Immunology* (Roit ed., Blackwell Scientific Publications, Palo Alto, 6th ed.), p. 181).

Agents other than Aβ peptides should induce an immunogenic response against one or more of the preferred segments of Aβ listed above (e.g., 1-10, 1-7, 1-3, and 3-7). Preferably, such agents induce an immunogenic response that is specifically directed to one of these segments without being directed to other segments of Aβ.

Random libraries of peptides or other compounds can also be screened for suitability. Combinatorial libraries can be produced for many types of compounds that can be synthesized in a step-by-step fashion. Such compounds include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds, benzodiazepines, oligomeric N-substituted glycines and oligocarbamates. Large combinatorial libraries of the compounds can be constructed by the encoded synthetic libraries (ESL) method described in Affymax, WO 95/12608, Affymax, WO 93/06121, Columbia University, WO 94/08051, Pharmacopeia, WO 95/35503 and Scripps, WO 95/30642 (each of which is incorporated by reference for all purposes). Peptide libraries can also be generated by phage display methods. See, e.g., Devlin, W0 91/18980.

Combinatorial libraries and other compounds are initially screened for suitability by determining their capacity to bind to antibodies or lymphocytes (B or T) known to be specific for Aβ or other amyloidogenic peptides. For example, initial screens can be performed with any polyclonal sera or monoclonal antibody to Aβ or a fragment thereof. Compounds can then be screened for binding to a specific epitope within Aβ (e.g., 1-10, 1-7, 1-3, 1-4, 1-5 and 3-7). Compounds can be tested by the same procedures described for mapping antibody epitope specificities. Compounds identified by such screens are then further analyzed for capacity to induce antibodies or reactive lymphocytes to Aβ or fragments thereof. For example, multiple dilutions of sera can be tested on microtiter plates that have been precoated with Aβ or a fragment thereof and a standard ELISA can be performed to test for reactive antibodies to Aβ or the fragment. Compounds can then be tested for prophylactic and therapeutic efficacy in transgenic animals predisposed to an amyloidogenic disease, as described in the Examples. Such animals include, for example, mice bearing a 717 mutation of APP described by Games et al., supra, and mice bearing a 670/671 Swedish mutation of APP such as described by McConlogue et al., US 5,612,486 and Hsiao et al., *Science* 274, 99 (1996); Staufenbiel et al., *Proc. Natl. Acad. Sci.* USA *94*, 13287-13292 (1997); Sturchler-Pierrat et al., *Proc. Natl. Acad. Sci. USA 94,* 13287-13292 (1997); Borchelt et al., *Neuron 19*, 939-945

(1997)). The same screening approach can be used on other potential agents analogs of Aβ and longer peptides including fragments of Aβ, described above.

2. Agents Inducing Passive Immune Response

Therapeutic agents of the invention also include antibodies that specifically bind to Aβ or other component of amyloid plaques. Such antibodies can be monoclonal or polyclonal. Some such antibodies bind specifically to the aggregated form of Aβ without binding to the dissociated form. Some bind specifically to the dissociated form without binding to the aggregated form. Some bind to both aggregated and dissociated forms. Some such antibodies bind to a naturally occurring short form of Aβ (i.e., Aβ39, 40 or 41) without binding to a naturally occurring long form of Aβ (i.e., Aβ42 and Aβ43). Some antibodies bind to a long form without binding to a short form. Some antibodies bind to Aβ without binding to full-length amyloid precursor protein. Antibodies used in therapeutic methods usually have an intact constant region or at least sufficient of the constant region to interact with an Fc receptor. Human isotype IgG1 is preferred because of it having highest affinity of human isotypes for the FcRI receptor on phagocytic cells. Bispecific Fab fragments can also be used, in which one arm of the antibody has specificity for Aβ, and the other for an Fc receptor. Some antibodies bind to Aβ with a binding affinity greater than or equal to about 10^6, 10^7, 10^8, 10^9, or 10^{10} M^{-1}.

Polyclonal sera typically contain mixed populations of antibodies binding to several epitopes along the length of Aβ. However, polyclonal sera can be specific to a particular segment of Aβ, such as Aβ1-10. Monoclonal antibodies bind to a specific epitope within Aβ that can be a conformational or nonconformational epitope. Prophylactic and therapeutic efficacy of antibodies can be tested using the transgenic animal model procedures described in the Examples. Preferred monoclonal antibodies bind to an epitope within residues 1-10 of Aβ (with the first N terminal residue of natural Aβ designated 1). Some preferred monoclonal antibodies bind to an epitope within amino acids 1-5, and some to an epitope within 5-10. Some preferred antibodies bind to epitopes within amino acids 1-3, 1-4, 1-5, 1-6, 1-7 or 3-7. Some preferred antibodies bind to an epitope starting at resides 1-3 and ending at residues 7-11 of Aβ. Less preferred antibodies include those binding to epitopes with residues 10-15, 15-20, 25-30, 10-20, 20, 30, or 10-25 of Aβ. It is recommended that such antibodies be screened for activity in the mouse model described in the Examples before use. For example, it has been found that certain antibodies to epitopes within residues 10-18, 16-24, 18-21 and 33-42 lack activity. In some methods, multiple monoclonal antibodies having binding specificities to different epitopes are used. Such antibodies can be administered sequentially or simultaneously. Antibodies to amyloid components other than Aβ can also be used. For example, antibodies can be directed to the amyloid associated protein synuclein.

When an antibody is said to bind to an epitope within specified residues, such as Aβ 1-5 for example, what is meant is that the antibody specifically binds to a polypeptide containing the specified residues (i.e., Aβ 1-5 in this an example). Such an antibody does not necessarily contact every residue within Aβ 1-5. Nor does every single amino acid substitution or deletion with in Aβ1-5 necessarily significantly affect binding affinity. Epitope specificity of an antibody can be determined, for example, by forming a phage display library in which different members display different subsequences of Aβ. The phage display library is then selected for members specifically binding to an antibody under test. A family of sequences is isolated. Typically, such a family contains a common core sequence, and varying lengths of flanking sequences in different members. The shortest core sequence showing specific binding to the antibody defines the epitope bound by the antibody. Antibodies can also be tested for epitope specificity in a competition assay with an antibody whose epitope specificity has already been determined. For example, antibodies that compete with the 3D6 antibody for binding to Aβ bind to the same or similar epitope as 3D6, i.e., within residues Aβ 1-5. Likewise antibodies that compete with the 10D5 antibody bind to the same or similar epitope, i.e, within residues Aβ 3-6. Screening antibodies for epitope specificity is a useful predictor of therapeutic efficacy. For example, an antibody determined to bind to an epitope within residues 1-7 of Aβ is likely to be effective in preventing and treating Alzheimer's disease.

Monoclonal or polyclonal antibodies that specifically bind to a preferred segment of Aβ without binding to other regions of Aβ have a number of advantages relative to monoclonal antibodies binding to other regions or polyclonal sera to intact Aβ. First, for equal mass dosages, dosages of antibodies that specifically bind to preferred segments contain a higher molar dosage of antibodies effective in clearing amyloid plaques. Second, antibodies specifically binding to preferred segments can induce a clearing response against amyloid deposits without inducing a clearing response against intact APP polypeptide, thereby reducing the potential for side effects.

i. General Characteristics of Immunoglobulins

The basic antibody structural unit is known to comprise a tetramer of subunits. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (*See generally, Fundamental Immunology* (Paul, W., ed., 2nd ed. Raven Press, N.Y., 1989), Ch. 7 (incorporated by reference in its entirety for all purposes).

The variable regions of each light/heavy chain pair form the antibody binding site. Thus, an intact antibody has two binding sites. Except in bifunctional or bispecific antibodies, the two binding sites are the same. The chains all exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. From N-terminal to C-terminal, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the definitions of Kabat, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987 and 1991), or Chothia & Lesk, *J. Mol. Biol.* 196:901-917 (1987); Chothia et al., *Nature* 342:878-883 (1989).

ii. Production of Nonhuman Antibodies

The production of non-human monoclonal antibodies, e.g., murine, guinea pig, primate, rabbit or rat, can be accomplished by, for example, immunizing the animal with Aβ. A longer polypeptide comprising Aβ or an immunogenic fragment of Aβ or anti-idiotypic antibodies to an antibody to Aβ can also be used. See Harlow & Lane, *Antibodies, A Laboratory Manual* (CSHP NY, 1988) (incorporated by reference for all purposes). Such an immunogen can be obtained from a natural source, by peptide synthesis or by recombinant expression. Optionally, the immunogen can be administered fused or otherwise complexed with a carrier protein, as described below. Optionally, the immunogen can be administered with an adjuvant. Several types of adjuvant can be used as described below. Complete Freund's adjuvant followed by incomplete adjuvant is preferred for immunization of laboratory animals. Rabbits or guinea pigs are typically used for making polyclonal antibodies. Mice are typically used for making monoclonal antibodies. Antibodies are screened for specific binding to Aβ. Optionally, antibodies are further screened for binding to a specific region of Aβ. The latter screening can be accomplished by determining binding of an antibody to a collection of deletion mutants of an Aβ peptide and determining which deletion mutants bind to the antibody. Binding can be assessed, for example, by Western blot or ELISA. The smallest fragment to show specific binding to the antibody defines the epitope of the antibody. Alternatively, epitope specificity can be determined by a competition assay is which a test and reference antibody compete for binding to Aβ. If the test and reference antibodies compete, then they bind to the same epitope or epitopes sufficiently proximal that binding of one antibody interferes with binding of the other. The preferred isotype for such antibodies is mouse isotype IgG2a or equivalent isotype in other species. Mouse isotype IgG2a is the equivalent of human isotype IgG1.

iii. Chimeric and Humanized Antibodies

Chimeric and humanized antibodies have the same or similar binding specificity and affinity as a mouse or other nonhuman antibody that provides the starting material for construction of a chimeric or humanized antibody. Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin gene segments belonging to different species. For example, the variable (V) segments of the genes from a mouse monoclonal antibody may be joined to human constant (C) segments, such as IgG1 and IgG4. Human isotype IgG1 is preferred. A typical chimeric antibody is thus a hybrid protein consisting of the V or antigen-binding domain from a mouse antibody and the C or effector domain from a human antibody.

Humanized antibodies have variable region framework residues substantially from a human antibody (termed an acceptor antibody) and complementarity determining regions substantially from a mouse-antibody, (referred to as the donor immunoglobulin). See , Queen et al., *Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989) and WO 90/07861, US 5,693,762, US 5,693,761, US 5,585,089, US 5,530,101 and Winter, US 5,225,539 (incorporated by reference in their entirety for all purposes). The constant region(s), if present, are also substantially or entirely from a human immunoglobulin. The human variable domains are usually chosen from human antibodies whose framework sequences exhibit a high degree of sequence identity with the murine variable region domains from which the CDRs were derived. The heavy and light chain variable region framework residues can be derived from the same or different human antibody sequences. The human antibody sequences can be the sequences of naturally occurring human antibodies or can be consensus sequences of several human antibodies. See Carter et al., WO 92/22653. Certain amino acids from the human variable region framework residues are selected for substitution based on their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is by modeling, examination of the characteristics of the amino acids at particular locations, or empirical observation of the effects of substitution or mutagenesis of particular amino acids.

For example, when an amino acid differs between a murine variable region framework residue and a selected human variable region framework residue, the human framework amino acid should usually be substituted by the equivalent framework amino acid from the mouse antibody when it is reasonably expected that the amino acid:

(1) noncovalently binds antigen directly,

(2) is adjacent to a CDR region,

(3) otherwise interacts with a CDR region (e.g. is within about 6 A of a CDR region), or

(4) participates in the VL-VH interface.

Other candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position. These amino acids can be substituted with amino acids from the equivalent position of the mouse donor antibody or from the equivalent positions of more typical human immunoglobulins. Other candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position.. The variable region frameworks of humanized immunoglobulins usually show at least 85% sequence identity to a human variable region framework sequence or consensus of such sequences.

iv. Human Antibodies

Human antibodies against Aβ are provided by a variety of techniques described below. Some human antibodies are selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody, such as one of the mouse monoclonals described in Example XI. Human antibodies can also be screened for a particular epitope specificity by using only a fragment of Aβ as the immunogen, and/or by screening antibodies against a collection of deletion mutants of Aβ. Human antibodies preferably have isotype specificity human IgG1.

(1) Trioma Methodology

The basic approach and an exemplary cell fusion partner, SPAZ-4, for use in this approach have been described by Oestberg et al., *Hybridoma* 2:361-367 (1983); Oestberg, U.S. Patent No. 4,634,664; and Engleman et al., US Patent 4,634,666 (each of which is incorporated by reference in its entirety for all purposes). The antibody-producing cell lines obtained by this method are called triomas, because they are descended from three cells--two human and one mouse. Initially, a mouse myeloma line is fused with a human B-lymphocyte to obtain a non-antibody-producing xenogeneic hybrid cell, such as the SPAZ-4 cell line described by Oestberg, supra. The xenogeneic cell is then fused with an immunized human B-lymphocyte to obtain an antibody-producing trioma cell line. Triomas have been found to produce antibody more stably than ordinary hybridomas made from human cells.

The immunized B-lymphocytes are obtained from the blood, spleen, lymph nodes or bone marrow of a human donor. If antibodies against a specific antigen or epitope are desired, it is preferable to use that antigen or epitope thereof for immunization. Immunization can be either in vivo or in vitro. For in vivo immunization, B cells are typically isolated from a human immunized with Aβ, a fragment thereof, larger polypeptide containing Aβ or fragment, or an anti-idiotypic antibody to an antibody to Aβ. In some methods, B cells are isolated from the same patient who is ultimately to be administered antibody therapy. For in vitro immunization, B-lymphocytes are typically exposed to antigen for a period of 7-14 days in a media such as RPMI-1640 (see Engleman, *supra*) supplemented with 10% human plasma.

The immunized B-lymphocytes are fused to a xenogeneic hybrid cell such as SPAZ-4 by well known methods. For example, the cells are treated with 40-50% polyethylene glycol of MW 1000-4000, at about 37 degrees C, for about 5-10 min. Cells are separated from the fusion mixture and propagated in media selective for the desired hybrids (e.g., HAT or AH). Clones secreting antibodies having the required binding specificity are identified by assaying the trioma culture medium for the ability to bind to Aβ or a fragment thereof. Triomas producing human antibodies having the desired specificity are subcloned by the limiting dilution technique and grown in vitro in culture medium. The trioma cell lines obtained are then tested for the ability to bind Aβ or a fragment thereof.

Although triomas are genetically stable they do not produce antibodies at very high levels. Expression levels can be increased by cloning antibody genes from the trioma into one or more expression vectors, and transforming the vector into standard mammalian, bacterial or yeast cell lines.

(2) Transgenic Non-Human Mammals

Human antibodies against Aβ can also be produced from non-human transgenic mammals having transgenes encoding at least a segment of the human immunoglobulin locus. Usually, the endogenous immunoglobulin locus of such transgenic mammals is functionally inactivated. Preferably, the segment of the human immunoglobulin locus includes unrearranged sequences of heavy and light chain components. Both inactivation of endogenous immunoglobulin genes and introduction of exogenous immunoglobulin genes can be achieved by targeted homologous recombination, or by introduction of YAC chromosomes. The transgenic mammals resulting from this process are capable of functionally rearranging the immunoglobulin component sequences, and expressing a repertoire of antibodies of various isotypes encoded by human immunoglobulin genes, without expressing endogenous immunoglobulin genes. The production and properties of mammals having these properties are described in detail by, e.g., Lonberg et al., WO93/12227 (1993); US 5,877,397, US 5,874,299, US 5,814,318, US 5,789,650, US 5,770,429, US 5,661,016, US 5,633,425, US 5,625,126, US 5,569,825, US 5,545,806, *Nature* 148, 1547-1553 (1994), *Nature Biotechnology* 14, 826 (1996), Kucherlapati, WO 91/10741 (1991) (each of which is incorporated by reference in its entirety for all purposes). Transgenic mice are particularly suitable. Anti-Aβ antibodies are obtained by immunizing a transgenic nonhuman mammal, such as described by Lonberg or Kucherlapati, *supra,* with Aβ or a fragment thereof. Monoclonal antibodies are prepared by, e.g., fusing B-cells from such mammals to suitable myeloma cell lines using conventional Kohler-Milstein technology. Human polyclonal antibodies can also be provided in the form of serum from humans immunized with an immunogenic agent. Optionally, such polyclonal antibodies can be concentrated by affinity purification using Aβ or other amyloid peptide as an affinity reagent.

(3) Phage Display Methods

A further approach for obtaining human anti-Aβ antibodies is to screen a DNA library from human B cells according to the general protocol outlined by Huse et al., *Science* 246:1275-1281 (1989). As described for trioma methodology, such B cells can be obtained from a human immunized with Aβ, fragments, longer polypeptides containing Aβ or fragments or anti-idiotypic antibodies. Optionally, such B cells are obtained from a patient who is ultimately to receive antibody treatment. Antibodies binding to Aβ or a fragment thereof are selected. Sequences encoding such antibodies (or a binding fragments) are then cloned and amplified. The protocol described by Huse is rendered more efficient in combination with phage-display technology. See, e.g., Dower et al., WO 91/17271 and McCafferty et al., WO 92/01047, US 5,877,218, US 5,871,907, US 5,858,657, US 5,837,242, US 5,733,743 and US 5,565,332 (each of which is incorporated by reference in its entirety for all purposes). In these methods, libraries of phage are produced in which members display different antibodies on their outer surfaces. Antibodies are usually displayed as Fv or Fab fragments. Phage displaying antibodies with a desired specificity are selected by affinity enrichment to an Aβ peptide or fragment thereof.

In a variation of the phage-display method, human antibodies having the binding specificity of a selected murine antibody can be produced. See Winter, WO 92/20791. In this method, either the heavy or light chain variable region of the selected murine antibody is used as a starting material. If, for example, a light chain variable region is selected as the starting material, a phage library is constructed in which members display the same light chain variable region (i.e., the murine starting material) and a different heavy chain variable region. The heavy chain variable regions are obtained from a library of rearranged human heavy chain variable regions. A phage showing strong specific binding for Aβ (e.g., at least 10^8 and preferably at least 10^9 M^{-1}) is selected. The human heavy chain variable region from this phage then serves as a starting material for constructing a further phage library. In this library, each phage displays the same heavy chain variable region (i.e., the region identified from the first display library) and a different light chain variable region. The light chain variable regions are obtained from a library of rearranged human variable light chain regions. Again, phage showing strong specific binding for Aβare selected. These phage display the variable regions of completely human anti-Aβ antibodies. These antibodies usually have the same or similar epitope specificity as the murine starting material.

v. Selection of Constant Region

The heavy and light chain variable regions of chimeric, humanized, or human antibodies can be linked to at least a portion of a human constant region. The choice of constant region depends, in part, whether antibody-dependent complement and/or cellular mediated toxicity is desired. For example, isotopes IgG1 and IgG3 have complement activity and isotypes IgG2 and IgG4 do not. Choice of isotype can also affect passage of antibody into the brain. Human isotype IgG1 is preferred. Light chain constant regions can be lambda or kappa. Antibodies can be expressed as tetramers containing two light and two heavy chains, as separate heavy chains, light chains, as Fab, Fab' F(ab')2, and Fv, or as single chain antibodies in which heavy and light chain variable domains are linked through a spacer.

vi. Expression of Recombinant Antibodies

Chimeric, humanized and human antibodies are typically produced by recombinant expression. Recombinant polynucleotide constructs typically include an expression control sequence operably linked to the coding sequences of antibody chains, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences are eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and the collection and purification of the crossreacting antibodies.

These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors contain selection markers, e.g., ampicillin-resistance or hygromycin-resistance, to permit detection of those cells transformed with the desired DNA sequences.

E. coli is one prokaryotic host particularly useful for cloning the DNA sequences of the present invention. Microbes, such as yeast are also useful for expression. Saccharomyces is a preferred yeast host, with suitable vectors having expression control sequences, an origin of replication, termination sequences and the like as desired. Typical promoters include 3-phosphoglycerate kinase and other glycolytic enzymes. Inducible yeast promoters include, among others, promoters from alcohol dehydrogenase, isocytochrome C, and enzymes responsible for maltose and galactose utilization.

Mammalian cells are a preferred host for expressing nucleotide segments encoding immunoglobulins or fragments thereof. See Winnacker, From Genes to Clones, (VCH Publishers, NY, 1987). A number of suitable host cell lines capable of secreting intact heterologous proteins have been developed in the art, and include CHO cell lines, various COS cell lines, HeLa cells, L cells and myeloma cell lines. Preferably, the cells are nonhuman. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al., *Immunol. Rev.* 89:49 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from endogenous genes, cytomegalovirus, SV40, adenovirus, bovine papillomavirus, and the like. See Co et al., *J. Immunol.* 148:1149 (1992).

Alternatively, antibody coding sequences can be incorporated in transgenes for introduction into the genome of a transgenic animal and subsequent expression in the milk of the transgenic animal (see, e.g., US 5,741,957, US 5,304,489, US 5,849,992). Suitable transgenes include coding sequences for light and/or heavy chains in operable linkage with a promoter and enhancer from a mammary gland specific gene, such as casein or beta lactoglobulin.

The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment, electroporation, lipofection, biolistics or viral-based transfection can be used for other cellular hosts. Other methods used to transform mammalian cells include the use of polybrene, protoplast fusion, liposomes, electroporation, and microinjection (see generally, Sambrook et al., supra). For production of transgenic animals, transgenes can be microinjected into fertilized oocytes, or can be incorporated into the genome of embryonic stem cells, and the nuclei of such cells transferred into enucleated oocytes.

Once expressed, antibodies can be purified according to standard procedures of the art, including HPLC purification, column chromatography, gel electrophoresis and the like (see generally, Scopes, *Protein Purification* (Springer-Verlag, NY, 1982)).

3. Carrier Proteins

Some agents for inducing an immune response contain the appropriate epitope for inducing an immune response against amyloid deposits but are too small to be immunogenic. In this situation, a peptide immunogen can be linked to a suitable carrier to help elicit an immune response. Suitable carriers include serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, or a toxoid from other pathogenic bacteria, such as diphtheria, *E. coli,* cholera, or *H. pylori*, or an attenuated toxin derivative. Other carriers include T-cell epitopes that bind to multiple MHC alleles, e.g., at least 75% of all human MHC alleles. Such carriers are sometimes known in the art as "universal T-cell epitopes." Examples of universal T-cell epitopes include:

Influenza Hemagluttinin: HA$_{307-319}$ P**K**Y**V**KQN**TLK**LAT

PADRE (common residues bolded) A**K**X**V**AAW**TLK**AAA

Malaria CS: T3 epitope EKKIAKMEKASSVFNV

Hepatitis B surface antigen: HBsAg$_{19-28}$ FFLLTRILTI

Heat Shock Protein 65: hsp65$_{153-171}$ DQSIGDLIAEAMDKVGNEG

bacille Calmette-Guerin QVHFQPLPPAVVKL

Tetanus toxoid: TT$_{830-844}$ QYIKANSKFIGITEL

Tetanus toxoid: TT$_{947-967}$ FNNFTVSFWLRVPKVSASHLE

HIV gp120 T1: KQIINMWQEVGKAMYA.

Other carriers for stimulating or enhancing an immune response include cytokines such as IL-1, IL-1 α and β peptides, IL-2, γINF, IL-10, GM-CSF, and chemokines, such as MIP1α and β and RANTES. Immunogenic agents can also be linked to peptides that enhance transport across tissues, as described in O'Mahony, WO 97/17613 and WO 97/17614.

Immunogenic agents can be linked to carriers by chemical crosslinking. Techniques for linking an immunogen to a carrier include the formation of disulfide linkages using N-succinimidyl-3-(2-pyridyl-thio) propionate (SPDP) and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (if the peptide lacks a sulfhydryl group, this can be provided by addition of a cysteine residue). These reagents create a disulfide linkage between themselves and peptide cysteine resides on one protein and an amide linkage through the ε -amino on a lysine, or other free amino group in other amino acids. A variety of such disulfide/amide-forming agents are described by *Immun. Rev.* 62, 185 (1982). Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thio-ether-forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid, 2-bromoacetic acid, and 2-iodoacetic acid, 4-(N-maleimido-methyl)cyclohexane-1-carboxylic acid. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxyl-2-nitro-4-sulfonic acid, sodium salt.

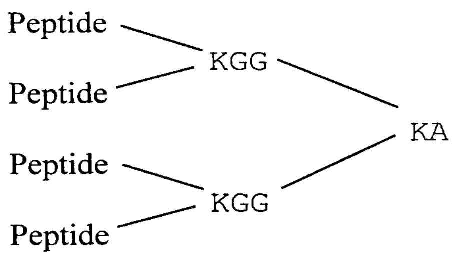
Immunogenic peptides can also be expressed as fusion proteins with carriers (i.e., heterologous peptides). The immunogenic peptide can be linked at its amino terminus, its carboxyl terminus, or both to a carrier. Optionally, multiple repeats of the immunogenic peptide can be present in the fusion protein. Optionally, an immunogenic peptide can be linked to multiple copies of a heterologous peptide, for example, at both the N and C termini of the peptide. Some carrier peptides serve to induce a helper T-cell response against the carrier peptide. The induced helper T-cells in turn induce a B-cell response against the immunogenic peptide linked to the carrier peptide.

Some agents of the invention comprise a fusion protein in which an N-terminal fragment of Aβ is linked at its C-terminus to a carrier peptide. In such agents, the N-terminal residue of the fragment of Aβ constitutes the N-terminal residue of the fusion protein. Accordingly, such fusion proteins are effective in inducing antibodies that bind to an epitope that requires the N-terminal residue of Aβ to be in free form. Some agents of the invention comprises a plurality of repeats of an N-terminal segment of Aβ linked at the C-terminus to one or more copy of a carrier peptide. The N-terminal fragment of Aβ incorporated into such fusion proteins sometimes begins at Aβ1-3 and ends at Aβ7-11. Aβ1-7, Aβ1-3, 1-4, 1-5, and 3-7 are preferred N-terminal fragment of Aβ. Some fusion proteins comprise different N-terminal segments of Aβ in tandem. For example, a fusion protein can comprise Aβ1-7 followed by Aβ1-3 followed by a heterologous peptide.

In some fusion proteins, an N-terminal segment of Aβ is fused at its N-terminal end to a heterologous carrier peptide. The same variety of N-terminal segments of Aβ can be used as with C-terminal fusions. Some fusion proteins comprise a heterologous peptide linked to the N-terminus of an N-terminal segment of Aβ, which is in turn linked to one or more additional N-terminal segments of Aβ in tandem.

Some examples of fusion proteins suitable for use in the invention are shown below. Some of these fusion proteins comprise segments of Aβ linked to tetanus toxoid epitopes such as described in US 5,196,512, EP 378,881 and EP 427,347. Some fusion proteins comprises segments of Aβ linked to carrier peptides described in US 5,736,142. Some heterologous peptides are universal T-cell epitopes. In some methods, the agent for administration is simply a single fusion protein with an Aβ segment linked to a heterologous segment in linear configuration. In some methods, the agent is multimer of fusion proteins represented by the formula 2^x, in which x is an integer from 1-5. Preferably x is 1, 2 or 3, with 2 being most preferred. When x is two, such a multimer has four fusion proteins linked in a preferred configuration referred to as MAP4 (see US 5,229,490). Epitopes of Aβ are underlined.

The MAP4 configuration is shown below, where branched structures are produced by initiating peptide synthesis at both the N terminal and side chain amines of lysine. Depending upon the number of times lysine is incorporated into the sequence and allowed to branch, the resulting structure will present multiple N termini. In this example, four identical N termini have been produced on the branched lysine-containing core. Such multiplicity greatly enhances the responsiveness of cognate B cells.



AN90549 (Aβ 1-7/Tetanus toxoid 830-844 in a MAP4 configuration):

DAEFRHDQYIKANSKFIGITEL

AN90550 (Aβ 1-7/Tetanus toxoid 947-967 in a MAP4 configuration):

DAEFRHDFNNFTVSFWLRVPKVSASHLE

AN90542 (Aβ 1-7/Tetanus toxoid 830-844 + 947-967 in a linear configuration):

DAEFRHDQYIKANSKFIGITELFNNFTVSFWLRVPKVSASHLE

AN90576: (Aβ 3-9)/Tetanus toxoid 830-844 in a MAP4 configuration):

EFRHDSGQYIKANSKFIGITEL

Peptide described in US 5,736,142 (all in linear configurations):

AN90562 (Aβ 1-7/ peptide) AKXVAAWTLKAAADAEFRHD

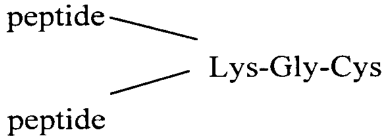
AN90543 (Aβ1-7 x 3/ peptide): DAEFRHDDAEFRHDDAEFRHDAKXVAAWTLKAAA

Other examples of fusion proteins (immunogenic epitope of Aβ bolded) include

AKXVAAWTLKAAA-**DAEFRHD-DAEFRHD-DAEFRHD**
DAEFRHD-AKXVAAWTLKAAA
DAEFRHD-ISQAVHAAHAEINEAGR
FRHDSGY-ISQAVHAAHAEINEAGR
EFRHDSG-ISQAVHAAHAEINEAGR
PKYVKQNTLKLAT-**DAEFRHD-DAEFRHD-DAEFRHD**
DAEFRHD-PKYVKQNTLKLAT-**DAEFRHD**
DAEFRHD-DAEFRHD-DAEFRHD-PKYVKQNTLKLAT

DAEFRHD-DAEFRHD-PKYVKQNTLKLAT
DAEFRHD-PKYVKQNTLKLAT-EKKIAKMEKASSVFNV-
QYIKANSKFIGITEL-FNNFTVSFWLRVPKVSASHLE-**DAEFRHD**
DAEFRHD-DAEFRHD-DAEFRHD-QYIKANSKFIGITEL-
FNNFTVSFWLRVPKVSASHLE
DAEFRHD-QYIKANSKFIGITEL**C**FNNFTVSFWLRVPKVSASHLE
DAEFRHD-QYIKANSKFIGITEL**C**FNNFTVSFWLRVPKVSASHLE-
DAEFRHD

DAEFRHD-QYIKANSKFIGITEL on a 2 branched resin



EQVTNVGGAISQAVHAAHAEINEAGR (Synuclein fusion protein in MAP-4 configuration)

The same or similar carrier proteins and methods of linkage can be used for generating immunogens to be used in generation of antibodies against Aβ for use in passive immunization. For example, Aβ or a fragment linked to a carrier can be administered to a laboratory animal in the production of monoclonal antibodies to Aβ.

4. Nucleic Acid Encoding Therapeutic Agents

Immune responses against amyloid deposits can also be induced by administration of nucleic acids encoding segments of Aβ peptide, and fragments thereof, other peptide immunogens, or antibodies and their component chains used for passive immunization. Such nucleic acids can be DNA or RNA. A nucleic acid segment encoding an immunogen is typically linked to regulatory elements, such as a promoter and enhancer, that allow expression of the DNA segment in the intended target cells of a patient. For expression in blood cells, as is desirable for induction of an immune response, promoter and enhancer elements from light or heavy chain immunoglobulin genes or the CMV major intermediate early promoter and enhancer are suitable to direct expression. The linked regulatory elements and coding sequences are often cloned into a vector. For administration of double-chain antibodies, the two chains can be cloned in the same or separate vectors.

A number of viral vector systems are available including retroviral systems *(see, e.g.*, Lawrie and Tumin, *Cur. Opin. Genet. Develop.* 3, 102-109 (1993)); adenoviral vectors (*see, e.g.*, Bett et al., *J. Virol.* 67, 5911 (1993)); adeno-associated virus vectors (see, e.g., Zhou et al., *J. Exp. Med.* 179, 1867 (1994)), viral vectors from the pox family including vaccinia virus and the avian pox viruses, viral vectors from the alpha virus genus such as those derived from Sindbis and Semliki Forest Viruses *(see, e.g.*, Dubensky et al., *J. Virol.* 70, 508-519 (1996)), Venezuelan equine encephalitis virus (see US 5,643,576) and rhabdoviruses, such as vesicular stomatitis virus (see WO 96/34625)and papillomaviruses (Ohe et al., *Human Gene Therapy* 6, 325-333 (1995); Woo et al., WO 94/12629 and Xiao & Brandsma, *Nucleic Acids. Res.* 24, 2630-2622 (1996)).

DNA encoding an immunogen, or a vector containing the same, can be packaged into liposomes. Suitable lipids and related analogs are described by US 5,208,036, 5,264,618, 5,279,833 and 5,283,185. Vectors and DNA encoding an immunogen can also be adsorbed to or associated with particulate carriers, examples of which include polymethyl methacrylate polymers and polylactides and poly(lactide-co-glycolides), see, e.g., McGee et al., *J. Micro Encap.* (1996).

Gene therapy vectors or naked DNA can be delivered in vivo by administration to an individual patient, typically by systemic administration (*e.g.*, intravenous, intraperitoneal, nasal, gastric, intradermal, intramuscular, subdermal, or intracranial infusion) or topical application (*see e.g.*, US 5,399,346). Such vectors can further include facilitating agents such as bupivacine (US 5,593,970). DNA can also be administered using a gene gun. See Xiao & Brandsma, *supra*. The DNA encoding an immunogen is precipitated onto the surface of microscopic metal beads. The microprojectiles are accelerated with a shock wave or expanding helium gas, and penetrate tissues to a depth of several cell layers. For example, The Accel™ Gene Delivery Device manufactured by Agacetus, Inc. Middleton WI is suitable. Alternatively, naked DNA can pass through skin into the blood stream simply by spotting the DNA onto skin with chemical or mechanical irritation (see WO 95/05853).

In a further variation, vectors encoding immunogens can be delivered to cells ex vivo, such as cells explanted from an individual patient (e.g., lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

III. Screening Antibodies for Clearing Activity

The invention provides methods of screening an antibody for activity in clearing an amyloid deposit or any other antigen, or associated biological entity, for which clearing activity is desired. To screen for activity against an amyloid deposit, a tissue sample from a brain of a patient with Alzheimer's disease or an animal model having characteristic Alzheimer's pathology is contacted with phagocytic cells bearing an Fc receptor, such as microglial cells, and the antibody under test in a medium in vitro. The pagocytic cells can be a primary culture or a cell line, such as BV-2, C8-B4, or THP-1. In some methods, the components are combined on a microscope slide to facilitate microscopic monitoring. In some methods, multiple reactions are performed in parallel in the wells of a microtiter dish. In such a format, a separate miniature microscope slide can be mounted in the separate wells, or a nonmicroscopic detection format, such as ELISA detection of Aβ can be used. Preferably, a series of measurements is made of the amount of amyloid deposit in the in vitro reaction mixture, starting from a baseline value before the reaction has proceeded, and one or more test values during the reaction. The antigen can be detected by staining, for example, with a fluorescently labelled antibody to Aβ or other component of amyloid plaques. The antibody used for staining may or may not be the same as the antibody being tested for clearing activity. A reduction relative to baseline during the reaction of the amyloid deposits indicates that the antibody under test has clearing activity. Such antibodies are likely to be useful in preventing or treating Alzheimer's and other amyloidogenic diseases.

Analogous methods can be used to screen antibodies for activity in clearing other types of biological entities. The assay can be used to detect clearing activity against virtually any kind of biological entity. Typically, the biological entity has some role in human or animal disease. The biological entity can be provided as a tissue sample or in isolated form. If provided as a tissue sample, the tissue sample is preferably unfixed to allow ready access to components of the tissue sample and to avoid perturbing the conformation of the components incidental to fixing. Examples of tissue samples that can be tested in this assay include cancerous tissue, precancerous tissue, tissue containing benign growths such as warts or moles, tissue infected with pathogenic microorganisms, tissue infiltrated with inflammatory cells, tissue bearing pathological matrices between cells (e.g., fibrinous pericarditis), tissue bearing aberrant antigens, and scar tissue. Examples of isolated biological entities that can be used include Aβ, viral antigens or viruses, proteoglycans, antigens of other pathogenic microorganisms, tumor antigens, and adhesion molecules. Such antigens can be obtained from natural sources, recombinant expression or chemical synthesis, among other means. The tissue sample or isolated biological entity is contacted with phagocytic cells bearing Fc receptors, such as monocytes or microglial cells, and an antibody to be tested in a medium. The antibody can be directed to the biological entity under test or to an antigen associated with the entity In the latter situation, the object is to test whether the biological entity is vicariously phagocytosed with the antigen. Usually, although not necessarily, the antibody and biological entity (sometimes with an associated antigen) are contacted with each other before adding the phagocytic cells. The concentration of the biological entity and/or the associated antigen, if present, remaining in the medium is then monitored. A reduction in the amount or concentration of antigen or the associated biological entity in the medium indicates the antibody has a clearing response against the antigen and/or associated biological entity in conjunction with the phagocytic cells (see, e.g., Example 14).

IV. PATIENTS AMENABLE TO TREATMENT

Patients amenable to treatment include individuals at risk of disease but not showing symptoms, as well as patients presently showing symptoms. In the case of Alzheimer's disease, virtually anyone is at risk of suffering from Alzheimer's disease if he or she lives long enough. Therefore, the present methods can be administered prophylactically to the general population without the need for any assessment of the risk of the subject patient. The present methods are especially useful for individuals who do have a known genetic risk of Alzheimer's disease. Such individuals include those having relatives who have experienced this disease, and those whose risk is determined by analysis of genetic or biochemical markers. Genetic markers of risk toward Alzheimer's disease include mutations in the APP gene, particularly mutations at position 717 and positions 670 and 671 referred to as the Hardy and Swedish mutations respectively (see Hardy, TINS, supra). Other markers of risk are mutations in the presenilin genes, PS1 and PS2, and ApoE4, family history of AD, hypercholesterolemia or atherosclerosis. Individuals presently suffering from Alzheimer's disease can be recognized from characteristic dementia, as well as the presence of risk factors described above. In addition, a number of diagnostic tests are available for identifying individuals who have

AD. These include measurement of CSF tau and Aβ42 levels. Elevated tau and decreased Aβ42 levels signify the presence of AD. Individuals suffering from Alzheimer's disease can also be diagnosed by ADRDA criteria as discussed in the Examples section.

In asymptomatic patients, treatment can begin at any age (e.g., 10, 20, 30). Usually, however, it is not necessary to begin treatment until a patient reaches 40, 50, 60 or 70. Treatment typically entails multiple dosages over a period of time. Treatment can be monitored by assaying antibody, or activated T-cell or B-cell responses to the therapeutic agent (e.g., Aβ peptide) over time. If the response falls, a booster dosage is indicated. In the case of potential Down's syndrome patients, treatment can begin antenatally by administering therapeutic agent to the mother or shortly after birth.

V. TREATMENT REGIMES

In prophylactic applications, pharmaceutical compositions or medicaments are administered to a patient susceptible to, or otherwise at risk of, Alzheimer’s disease in an amount sufficient to eliminate or reduce the risk, lessen the severity, or delay the outset of the disease, including biochemical, histologic and/or behavioral symptoms of the disease, its complications and intermediate pathological phenotypes presenting during development of the disease. In therapeutic applications, compositions or medicants are administered to a patient suspected of, or already suffering from such a disease in an amount sufficient to cure, or at least partially arrest, the symptoms of the disease (biochemical, histologic and/or behavioral), including its complications and intermediate pathological phenotypes in development of the disease. In some methods, administration of agent reduces or eliminates myocognitive impairment in patients that have not yet developed characteristic Alzheimer’s pathology. An amount adequate to accomplish therapeutic or prophylactic treatment is defined as a therapeutically- or prophylactically-effective dose. In both prophylactic and therapeutic regimes, agents are usually administered in several dosages until a sufficient immune response has been achieved. Typically, the immune response is monitored and repeated dosages are given if the immune response starts to wane.

Effective doses of the compositions of the present invention, for the treatment of the above described conditions vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human but nonhuman mammals including transgenic mammals can also be treated. Treatment dosages need to be titrated to optimize safety and efficacy. The amount of immunogen depends on whether adjuvant is also administered, with higher dosages being required in the absence of adjuvant. The amount of an immunogen for administration sometimes varies from 1-500 μg per patient and more usually from 5-500 μg per injection for human administration. Occasionally, a higher dose of 1-2 mg per injection is used. Typically about 10, 20, 50 or 100 μg is used for each human injection. The mass of immunogen also depends on the mass ratio of immunogenic epitope within the immunogen to the mass of immunogen as a whole. Typically, 10^{-3} to 10^{-5} micromoles of immunogenic epitope are used for microgram of immunogen. The timing of injections can vary significantly from once a day, to once a year, to once a decade. On any given day that a dosage of immunogen is given, the dosage is greater than 1 μg/patient and usually greater than 10 μg/ patient if adjuvant is also administered, and greater than 10 μg/patient and usually greater than 100 μg/patient in the absence of adjuvant. A typical regimen consists of an immunization followed by booster injections at time intervals, such as 6 week intervals. Another regimen consists of an immunization followed by booster injections 1, 2 and 12 months later. Another regimen entails an injection every two months for life. Alternatively, booster injections can be on an irregular basis as indicated by monitoring of immune response.

For passive immunization with an antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages can be 1 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg. An exemplary treatment regime entails administration once per every two weeks or once a month or once every 3 to 6 months. In some methods, two or more monoclonal antibodies with different binding specificities are administered simultaneously, in which case the dosage of each antibody administered falls within the ranges indicated. Antibody is usually administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of antibody to Aβ in the patient. In some methods, dosage is adjusted to achieve a plasma antibody concentration of 1–1000 ug/ml and in some methods 25 – 300 ug/ml. Alternatively, antibody can be administered as a sustained release formulation, in which case less frequent administration is required.

Dosage and frequency vary depending on the half-life of the antibody in the patient. In general, human antibodies show the longest half life, followed by humanized antibodies, chimeric antibodies, and nonhuman antibodies. The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, a relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patent can be administered a prophylactic regime.

Doses for nucleic acids encoding immunogens range from about 10 ng to 1 g, 100 ng to 100 mg, 1 μg to 10 mg, or 30-300 μg DNA per patient. Doses for infectious viral vectors vary from 10-100, or more, virions per dose.

Agents for inducing an immune response can be administered by parenteral, topical, intravenous, oral, subcutaneous, intraarterial, intracranial, intraperitoneal, intranasal or intramuscular means for prophylactic and/or therapeutic treatment. The most typical route of administration of an immunogenic agent is subcutaneous although other routes can be equally effective. The next most common route is intramuscular injection. This type of injection is most typically performed in the arm or leg muscles. In some methods, agents are injected directly into a particular tissue where deposits have accumulated, for example intracranial injection. Intramuscular injection on intravenous infusion are preferred for administration of antibody. In some methods, particular therapeutic antibodies are injected directly into the cranium. In some methods, antibodies are administered as a sustained release composition or device, such as a Medipad™ device.

Agents of the invention can optionally be administered in combination with other agents that are at least partly effective in treatment of amyloidogenic disease. In the case of Alzheimer's and Down's syndrome, in which amyloid deposits occur in the brain, agents of the invention can also be administered in conjunction with other agents that increase passage of the agents of the invention across the blood-brain barrier.

Immunogenic agents of the invention, such as peptides, are sometimes administered in combination with an adjuvant. A variety of adjuvants can be used in combination with a peptide, such as Aβ, to elicit an immune response. Preferred adjuvants augment the intrinsic response to an immunogen without causing conformational changes in the immunogen that affect the qualitative form of the response. Preferred adjuvants include aluminum hydroxide and aluminum phosphate, 3 De-O-acylated monophosphoryl lipid A (MPL™) (see GB 2220211 (RIBI ImmunoChem Research Inc., Hamilton, Montana, now part of Corixa). Stimulon™ QS-21 is a triterpene glycoside or saponin isolated from the bark of the Quillaja Saponaria Molina tree found in South America (see Kensil et al., in *Vaccine Design: The Subunit and Adjuvant Approach* (eds. Powell & Newman, Plenum Press, NY, 1995); US Patent No. 5,057,540),(Aquila BioPharmaceuticals, Framingham, MA). Other adjuvants are oil in water emulsions (such as squalene or peanut oil), optionally in combination with immune stimulants, such as monophosphoryl lipid A (see Stoute et al., *N. Engl. J. Med.* 336, 86-91 (1997)). Another adjuvant is CpG (WO 98/40100). Alternatively, Aβ can be coupled to an adjuvant. However, such coupling should not substantially change the conformation of Aβ so as to affect the nature of the immune response thereto. Adjuvants can be administered as a component of a therapeutic composition with an active agent or can be administered separately, before, concurrently with, or after administration of the therapeutic agent.

A preferred class of adjuvants is aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate. Such adjuvants can be used with or without other specific immunostimulating agents such as MPL or 3-DMP, QS-21, polymeric or monomeric amino acids such as polyglutamic acid or polylysine. Another class of adjuvants is oil-in-water emulsion formulations. Such adjuvants can be used with or without other specific immunostimulating agents such as muramyl peptides (*e.g.*, N-acetylmuramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), N-acetylglucsaminyl-N-acetylmuramyl-L-Al-D-isoglu-L-Ala-dipalmitoxy propylamide (DTP-DPP) theramideTM), or other bacterial cell wall components. Oil-in-water emulsions include (a) MF59 (WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton MA), (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5%

pluronic-blocked polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi ImmunoChem, Hamilton, MT) containing 2% squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphoryllipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™). Another class of preferred adjuvants is saponin adjuvants, such as Stimulon™ (QS-21, Aquila, Framingham, MA) or particles generated therefrom such as ISCOMs (immunostimulating complexes) and ISCOMATRIX. Other adjuvants include Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA). Other adjuvants include cytokines, such as interleukins (IL-1, IL-2, and IL-12), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF).

An adjuvant can be administered with an immunogen as a single composition, or can be administered before, concurrent with or after administration of the immunogen. Immunogen and adjuvant can be packaged and supplied in the same vial or can be packaged in separate vials and mixed before use. Immunogen and adjuvant are typically packaged with a label indicating the intended therapeutic application. If immunogen and adjuvant are packaged separately, the packaging typically includes instructions for mixing before use. The choice of an adjuvant and/or carrier depends on the stability of the immunogenic formulation containing the adjuvant, the route of administration, the dosing schedule, the efficacy of the adjuvant for the species being vaccinated, and, in humans, a pharmaceutically acceptable adjuvant is one that has been approved or is approvable for human administration by pertinent regulatory bodies. For example, Complete Freund's adjuvant is not suitable for human administration. Alum, MPL and QS-21 are preferred. Optionally, two or more different adjuvants can be used simultaneously. Preferred combinations include alum with MPL, alum with QS-21, MPL with QS-21, and alum, QS-21 and MPL together. Also, Incomplete Freund's adjuvant can be used (Chang *et al.*, *Advanced Drug Delivery Reviews* 32, 173-186 (1998)), optionally in combination with any of alum, QS-21, and MPL and all combinations thereof.

Agents of the invention are often administered as pharmaceutical compositions comprising an active therapeutic agent, i.e., and a variety of other pharmaceutically acceptable components. See *Remington's Pharmaceutical Science* (15th ed., Mack Publishing Company, Easton, Pennsylvania, 1980). The preferred form depends on the intended mode of administration and therapeutic application. The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like.

Pharmaceutical compositions can also include large, slowly metabolized macromolecules such as proteins, polysaccharides such as chitosan, polylactic acids, polyglycolic acids and copolymers (such as latex functionalized sepharose(TM), agarose, cellulose, and the like), polymeric amino acids, amino acid copolymers, and lipid aggregates (such as oil droplets or liposomes). Additionally, these carriers can function as immunostimulating agents (i.e., adjuvants).

For parenteral administration, agents of the invention can be administered as injectable dosages of a solution or suspension of the substance in a physiologically acceptable diluent with a pharmaceutical carrier that can be a sterile liquid such as water oils, saline, glycerol, or ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, surfactants, pH buffering substances and the like can be present in compositions. Other components of pharmaceutical compositions are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general, glycols such as propylene glycol or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions. Antibodies can be administered in the form of a depot injection or implant preparation which can be formulated in such a manner as to permit a sustained release of the active ingredient. An exemplary composition comprises monoclonal antibody at 5 mg/mL, formulated in aqueous buffer consisting of 50 mM L-histidine, 150 mM NaCl, adjusted to pH 6.0 with HCl.

Typically, compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The preparation also can be emulsified or encapsulated in liposomes or micro particles such as polylactide, polyglycolide, or copolymer for enhanced adjuvant effect, as discussed above (see Langer, *Science* 249, 1527 (1990) and Hanes, *Advanced Drug Delivery Reviews* 28, 97-119 (1997). The agents of this invention can be administered in the form of a depot injection or implant preparation which can be formulated in such a manner as to permit a sustained or pulsatile release of the active ingredient.

Additional formulations suitable for other modes of administration include oral, intranasal, and pulmonary formulations, suppositories, and transdermal applications.

For suppositories, binders and carriers include, for example, polyalkylene glycols or triglycerides; such suppositories can be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include excipients, such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, and magnesium carbonate. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

Topical application can result in transdermal or intradermal delivery. Topical administration can be facilitated by co-administration of the agent with cholera toxin or detoxified derivatives or subunits thereof or other similar bacterial toxins (See Glenn *et al., Nature* 391, 851 (1998)). Co-administration can be achieved by using the components as a mixture or as linked molecules obtained by chemical crosslinking or expression as a fusion protein.

Alternatively, transdermal delivery can be achieved using a skin path or using transferosomes (Paul *et al., Eur. J. Immunol.* 25, 3521-24 (1995); Cevc et al., *Biochem. Biophys. Acta* 1368, 201-15 (1998)).

VI. Methods of Diagnosis

The invention provides methods of detecting an immune response against Aβ peptide in a patient suffering from or susceptible to Alzheimer's disease. The methods are particularly useful for monitoring a course of treatment being administered to a patient. The methods can be used to monitor both therapeutic treatment on symptomatic patients and prophylactic treatment on asymptomatic patients. The methods are useful for monitoring both active immunization (e.g., antibody produced in response to administration of immunogen) and passive immunization (e.g., measuring level of administered antibody).

1. Active Immunization

Some methods entail determining a baseline value of an immune response in a patient before administering a dosage of agent, and comparing this with a value for the immune response after treatment. A significant increase (i.e., greater than the typical margin of experimental error in repeat measurements of the same sample, expressed as one standard deviation from the mean of such measurements) in value of the immune response signals a positive treatment outcome (i.e., that administration of the agent has achieved or augmented an immune response). If the value for immune response does not change significantly, or decreases, a negative treatment outcome is indicated. In general, patients undergoing an initial course of treatment with an immunogenic agent are expected to show an increase in immune response with successive dosages, which eventually reaches a plateau. Administration of agent is generally continued while the immune response is increasing. Attainment of the plateau is an indicator that the administered of treatment can be discontinued or reduced in dosage or frequency.

In other methods, a control value (i.e., a mean and standard deviation) of immune response is determined for a control population. Typically the individuals in the control population have not received prior treatment. Measured values of immune response in a patient after administering a therapeutic agent are then compared with the control value. A significant increase relative to the control value (e.g., greater than one standard deviation from the mean) signals a positive treatment outcome. A lack of significant increase or a decrease signals a negative treatment outcome. Administration of agent is generally continued while the immune response is increasing relative to the control value. As before, attainment of a plateau relative to control values in an indicator that the administration of treatment can be discontinued or reduced in dosage or frequency.

In other methods, a control value of immune response (*e.g.*, a mean and standard deviation) is determined from a control population of individuals who have undergone treatment with a therapeutic agent and whose immune responses have plateaued in response to treatment. Measured values of immune response in a patient are compared with the control value. If the measured level in a patient is not significantly different (*e.g.*, more than one standard deviation) from the control value, treatment can be discontinued. If the level in a patient is significantly below the control value, continued administration of agent is warranted. If the level in the patient persists below the control value, then a change in treatment regime, for example, use of a different adjuvant may be indicated.

In other methods, a patient who is not presently receiving treatment but has undergone a previous course of treatment is monitored for immune response to determine whether a resumption of treatment is required. The measured value of immune response in the patient can be compared with a value of immune response previously achieved in the patient after a previous course of treatment. A significant decrease relative to the previous measurement (i.e., greater than a typical margin of error in repeat measurements of the same sample) is an indication that treatment can be resumed. Alternatively, the value measured in a patient can be compared with a control value (mean plus standard deviation) determined in a population of patients after undergoing a course of treatment. Alternatively, the measured value in a patient can be compared with a control value in populations of prophylactically treated patients who remain free of symptoms of disease, or populations of therapeutically treated patients who show amelioration of disease characteristics. In all of these cases, a significant decrease relative to the control level (i.e., more than a standard deviation) is an indicator that treatment should be resumed in a patient.

The tissue sample for analysis is typically blood, plasma, serum, mucous or cerebrospinal fluid from the patient. The sample is analyzed for indication of an immune response to any form of Aβ peptide, typically Aβ42. The immune response can be determined from the presence of, e.g., antibodies or T-cells that specifically bind to Aβ peptide. ELISA methods of detecting antibodies specific to Aβ are described in the Examples section. Methods of detecting reactive T-cells have been described above (see Definitions). In some methods, the immune response is determined using a clearing assay, such as described in Section III above. In such methods, a tissue sample from a patient being tested is contacted with amyloid deposits (e.g., from a PDAPP mouse) and phagocytic cells bearing Fc receptors. Subsequent clearing of the amyloid deposit is then monitored. The existence and extent of clearing response provides an indication of the existence and level of antibodies effective to clear Aβ in the tissue sample of the patient under test.

2. Passive Immunization

In general, the procedures for monitoring passive immunization are similar to those for monitoring active immunization described above. However, the antibody profile following passive immunization typically shows an immediate peak in antibody concentration followed by an exponential decay. Without a further dosage, the decay approaches pretreatment levels within a period of days to months depending on the half-life of the antibody administered. For example the half-life of some human antibodies is of the order of 20 days.

In some methods, a baseline measurement of antibody to Aβ in the patient is made before administration, a second measurement is made soon thereafter to determine the peak antibody level, and one or more further measurements are made at intervals to monitor decay of antibody levels. When the level of antibody has declined to baseline or a predetermined percentage of the peak less baseline *(e.g.*, 50%, 25% or 10%), administration of a further dosage of antibody is administered. In some methods, peak or subsequent measured levels less background are compared with reference levels previously determined to constitute a beneficial prophylactic or therapeutic treatment regime in other patients. If the measured antibody level is significantly less than a reference level (e.g., less than the mean minus one standard deviation of the reference value in population of patients benefiting from treatment) administration of an additional dosage of antibody is indicated.

3. Diagnostic Kits

The invention further provides diagnostic kits for performing the diagnostic methods described above. Typically, such kits contain an agent that specifically binds to antibodies to Aβ. The kit can also include a label. For detection of antibodies to Aβ, the label is typically in the form of labelled anti-idiotypic antibodies. For detection of antibodies, the agent can be supplied prebound to a solid phase, such as to the wells of a microtiter dish. Kits also typically contain labeling providing directions for use of the kit. The labeling may also include a chart or other correspondence regime correlating levels of measured label with levels of antibodies to Aβ. The term labeling refers to any written or recorded material that is attached to, or otherwise accompanies a kit at any time during its manufacture, transport, sale or use. For example, the term labeling encompasses advertising leaflets and brochures, packaging materials, instructions, audio or video cassettes, computer discs, as well as writing imprinted directly on kits.

The invention also provides diagnostic kits for performing in vivo imaging. Such kits typically contain an antibody binding to an epitope of Aβ, preferably within residues 1-10. Preferably, the antibody is labelled or a secondary labeling reagent is included in the kit. Preferably, the kit is labelled with instructions for performing an in vivo imaging assay.

VII. *In Vivo* Imaging

The invention provides methods of *in vivo* imaging amyloid deposits in a patient. Such methods are useful to diagnose or confirm diagnosis of Alzheimer's disease, or susceptibility thereto. For example, the methods can be used on a patient presenting with symptoms of dementia. If the patient has abnormal amyloid deposits, then the patient is likely suffering from Alzheimer's disease. The methods can also be used on asymptomatic patients. Presence of abnormal deposits of amyloid indicates susceptibility to future symptomatic disease. The methods are also useful for monitoring disease progression and/or response to treatment in patients who have been previously diagnosed with Alzheimer's disease.

The methods work by administering a reagent, such as antibody, that binds to Aβ to the patient, and then detecting the agent after it has bound. Preferred antibodies bind to Aβ deposits in a patient without binding to full length APP polypeptide. Antibodies binding to an epitope of Aβ within amino acids 1-10 are particularly preferred. In some methods, the antibody binds to an epitope within amino acids 7-10 of Aβ. Such antibodies typically bind without inducing a substantial clearing response. In other methods, the antibody binds to an epitope within amino acids 1-7 of Aβ. Such antibodies typically bind and induce a clearing response to Aβ. However, the clearing response can be avoided by using antibody fragments lacking a full length constant region, such as Fabs. In some methods, the same antibody can serve as both a treatment and diagnostic reagent. In general, antibodies binding to epitopes C-terminal of residue 10 of Aβdo not show as strong signal as antibodies binding to epitopes within residues 1-10, presumably because the C-terminal epitopes are inaccessible in amyloid deposits. Accordingly, such antibodies are less preferred.

Diagnostic reagents can be administered by intravenous injection into the body of the patient, or directly into the brain by intracranial injection or by drilling a hole through the skull. The dosage of reagent should be within the same ranges as for treatment methods. Typically, the reagent is labelled, although in some methods, the primary reagent with affinity for Aβ is unlabelled and a secondary labeling agent is used to bind to the primary reagent. The choice of label depends on the means of detection. For example, a fluorescent label is suitable for optical detection. Use of paramagnetic labels is suitable for tomographic detection without surgical intervention. Radioactive labels can also be detected using PET or SPECT.

Diagnosis is performed by comparing the number, size and/or intensity of labelled loci to corresponding base line values. The base line values can represent the mean levels in a population of undiseased individuals. Base line values can also represent previous levels determined in the same patient. For example, base line values can be determined in a patient before beginning treatment, and measured values thereafter compared with the base line values. A decrease in values relative to base line signals a positive response to treatment.

EXAMPLES

I. PROPHYLACTIC EFFICACY OF Aβ AGAINST AD

These examples describe administration of Aβ42 peptide to transgenic mice overexpressing APP with a mutation at position 717 ($APP_{717V \rightarrow F}$) that predisposes them to develop Alzheimer's-like neuropathology. Production and characteristics of these mice (PDAPP mice) is described in Games et al., *Nature*, *supra*. These animals, in their heterozygote form, begin to deposit Aβ at six months of age forward. By fifteen months of age they exhibit levels of Aβ deposition equivalent to that seen in Alzheimer's disease. PDAPP mice were injected with aggregated $A\beta_{42}$ (aggregated $A\beta_{42}$) or phosphate buffered saline. Aggregated $A\beta_{42}$ was chosen because of its ability to induce antibodies to multiple epitopes of Aβ.

A. METHODS

1. Source of Mice

Thirty PDAPP heterogenic female mice were randomly divided into the following groups: 10 mice to be injected with aggregated Aβ42 (one died in transit), 5 mice to be injected with PBS/adjuvant or PBS, and 10 uninjected controls. Five mice were injected with peptides derived from the sequence of serum amyloid protein (SAP).

2. Preparation of Immunogens

Preparation of aggregated Aβ42: two milligrams of Aβ42 (US Peptides Inc, lot K-42-12) was dissolved in 0.9 ml water and made up to 1 ml by adding 0.1 ml 10 x PBS. This was vortexed and allowed to incubate overnight 37° C, under which conditions the peptide aggregated. Any unused Aβ was stored as a dry lyophilized powder at -20° C until the next injection.

3. Preparation of Injections

For each injection, 100 μg of aggregated Aβ42 in PBS per mouse was emulsified 1:1 with Complete Freund's adjuvant (CFA) in a final volume of 400 μl emulsion for the first immunization, followed by a boost of the same amount of immunogen in Incomplete Freund's adjuvant (IFA) at 2 weeks. Two additional doses in IFA were given at monthly intervals. The subsequent immunizations were done at monthly intervals in 500 μl of PBS. Injections were delivered intraperitoneally (i.p.).

PBS injections followed the same schedule and mice were injected with a 1:1 mix of PBS/ Adjuvant at 400 μl per mouse, or 500 μl of PBS per mouse. SAP injections likewise followed the same schedule using a dose of 100 μg per injection.

4. Titration of Mouse Bleeds, Tissue Preparation and Immunohistochemistry

The above methods are described infra in General Materials and Methods.

B. RESULTS

PDAPP mice were injected with either aggregated Aβ42 (aggregated Aβ42), SAP peptides, or phosphate buffered saline. A group of PDAPP mice were also left as uninjected, positive controls. The titers of the mice to aggregated Aβ42 were monitored every other month from the fourth boost until the mice were one year of age. Mice were sacrificed at 13 months. At all time points examined, eight of the nine aggregated Aβ42 mice developed a high antibody titer, which remained high throughout the series of injections (titers greater than 1/10000). The ninth mouse had a low, but measurable titer of approximately 1/1000 (Figure 1, Table 1). SAPP-injected mice had titers of 1:1,000 to 1:30,000 for this immunogen with only a single mouse exceeding 1:10,0000.

The PBS-treated mice were titered against aggregated Aβ42 at six, ten and twelve months. At a 1/100 dilution the PBS mice, when titered against aggregated Aβ42, only exceeded 4 times background at one data point, otherwise, they were less than 4 times background at all time points (Table 1). The SAP-specific response was negligible at these time points with all titers less than 300.

Seven out of the nine mice in the aggregated Aβ1-42 treated group had no detectable amyloid in their brains. In contrast, brain tissue from mice in the SAP and PBS groups contained numerous amyloid deposits in the hippocampus, as well as in the frontal and cingulate cortices. The pattern of deposition was similar to that of untreated controls, with characteristic involvement of vulnerable subregions, such as the outer molecular layer of the hippocampal dentate gyrus. One mouse from the Aβ 1-42-injected group had a greatly reduced amyloid burden, confined to the hippocampus. An isolated plaque was identified in another Aβ 1-42-treated mouse.

Quantitative image analyses of the amyloid burden in the hippocampus verified the dramatic reduction achieved in the Aβ42(AN1792)-treated animals (Fig. 2). The median values of the amyloid burden for the PBS group (2.22%), and for the untreated control group (2.65%) were significantly greater than for those immunized with AN1792 (0.00%, p=0.0005). In contrast, the median value for the group immunized with SAP peptides (SAPP) was 5.74%. Brain tissue from the untreated, control mice contained numerous Aβ amyloid deposits visualized with the Aβ-specific monoclonal antibody (mAb) 3D6 in the hippocampus, as well as in the retrosplenial cortex. A similar pattern of amyloid deposition was also seen in mice immunized with SAPP or PBS (Fig. 2). In addition, in these latter three groups there was a characteristic involvement of vulnerable subregions of the brain classically seen in AD, such as the outer molecular layer of the hippocampal dentate gyrus, in all three of these groups.

The brains that contained no Aβ deposits were also devoid of neuritic plaques that are typically visualized in PDAPP mice with the human APP antibody 8E5. All of brains from the remaining groups (SAP-injected, PBS and uninjected mice) had numerous neuritic plaques typical of untreated PDAPP mice. A small number of neuritic plaques were present in one mouse treated with AN1792, and a single cluster of dystrophic neurites was found in a second mouse treated with AN1792. Image analyses of the hippocampus, and shown in Fig. 3, demonstrated the virtual elimination of dystrophic neurites in AN1792-treated mice (median 0.00%) compared to the PBS recipients (median 0.28%, p = 0.0005).

Astrocytosis characteristic of plaque-associated inflammation was also absent in the brains of the Aβ1-42 injected group. The brains from the mice in the other groups contained abundant and clustered GFAP-positive astrocytes typical of Aβ plaque-associated gliosis. A subset of the GFAP-reacted slides were counter-stained with Thioflavin S to localize the Aβ deposits. The GFAP-positive astrocytes were associated with Aβ plaques in the SAP, PBS and untreated controls. No such association was found in the plaque-negative Aβ1-42 treated mice, while minimal plaque-associated gliosis was identified in one mouse treated with AN1792.

Image analyses, shown in Fig. 4 for the retrosplenial cortex, verified that the reduction in astrocytosis was significant with a median value of 1.56% for those treated with AN1792 versus median values greater than 6% for groups immunized with SAP peptides, PBS or untreated (p=0.0017)

Evidence from a subset of the Aβ1-42- and PBS-injected mice indicated plaque-associated MHC II immunoreactivity was absent in the Aβl-42 injected mice, consistent with lack of an Aβ-related inflammatory response.

Sections of the mouse brains were also reacted with a mAb specific with a monoclonal antibody specific for MAC-1, a cell surface protein. MAC-1 (CD11b) is an integrin family member and exists as a heterodimer with CD18. The CD11b/CD18 complex is present on monocytes, macrophages, neutrophils and natural killer cells (Mak and Simard). The resident MAC-1-reactive cell type in the brain is likely to be microglia based on similar phenotypic morphology in MAC-1 immunoreacted sections. Plaque-associated MAC-1 labeling was lower in the brains of mice treated with AN1792 compared to the PBS control group, a finding consistent with the lack of an Aβ-induced inflammatory response.

C. CONCLUSION

The lack of Aβ plaques and reactive neuronal and gliotic changes in the brains of the Aβ1-42-injected mice indicate that no or extremely little amyloid was deposited in their brains, and pathological consequences, such as gliosis and neuritic pathology, were absent. PDAPP mice treated with Aβ1-42 show essentially the same lack of pathology as control nontransgenic mice. Therefore, Aβ1-42 injections are highly effective in the prevention of deposition or clearance of human Aβ from brain tissue, and elimination of subsequent neuronal and inflammatory degenerative changes. Thus, administration of Aβ peptide can have both preventative and therapeutic benefit in prevention of AD.

II. DOSE RESPONSE STUDY

Groups of five-week old, female Swiss Webster mice (N=6 per group) were immunized with 300, 100, 33, 11, 3.7, 1.2, 0.4, or 0.13 ug of Aβ formulated in CFA/IFA administered intraperitoneally. Three doses were given at biweekly intervals followed by a fourth dose one month later. The first dose was emulsified with CFA and the remaining doses were emulsified with IFA. Animals were bled 4-7 days following each immunization starting after the second dose for measurement of antibody titers. Animals in a subset of three groups, those immunized with 11, 33, or 300 μg of antigen, were additionally bled at approximately monthly intervals for four months following the fourth immunization to monitor the decay of the antibody response across a range of doses of immunogenic formulations. These animals received a final fifth immunization at seven months after study initiation. They were sacrificed one week later to measure antibody responses to AN1792 and to perform toxicological analyses.

A declining dose response was observed from 300 to 3.7 μg with no response at the two lowest doses. Mean antibody titers are about 1:1000 after 3 doses and about 1:10,000 after 4 doses of 11-300 μg of antigen (see Fig. 5).

Antibody titers rose dramatically for all but the lowest dose group following the third immunization with increases in GMTs ranging from 5- to 25-fold. Low antibody responses were then detectable for even the 0.4 μg recipients. The 1.2 and 3.7 μg groups had comparable titers with GMTs of about 1000 and the highest four doses clustered together with GMTs of about 25,000, with the exception of the 33 μg dose group with a lower GMT of 3000. Following the fourth immunization, the titer increase was more modest for most groups. There was a clear dose response across the lower antigen dose groups from 0.14 μg to 11 μg ranging from no detectable antibody for recipients of 0.14 μg to a GMT of 36,000 for recipients of 11 μg. Again, titers for the four highest dose groups of 11 to 300 μg clustered together. Thus following two immunizations, the antibody titer was dependent on the antigen dose across the broad range from 0.4 to 300 μg. By the third immunization, titers of the highest four doses were all comparable and they remained at a plateau after an additional immunization.

One month following the fourth immunization, titers were 2- to 3-fold higher in the 300 μg group than those measured from blood drawn five days following the immunization (Fig. 6). This observation suggests that the peak anamnestic antibody response occurred later than 5 days post-immunization. A more modest (50%) increase was seen at this time in the 33 μg group. In the 300 μg dose group at two months following the last dose, GMTs declined steeply by about 70%. After another month, the decline was less steep at 45% (100 μg) and about 14% for the 33 and 11 μg doses. Thus, the rate of decline in circulating antibody titers following cessation of immunization appears to be biphasic with a steep decline the first month following peak response followed by a more modest rate of decrease thereafter.

The antibody titers and the kinetics of the response of these Swiss Webster mice are similar to those of young heterozygous PDAPP transgenic mice immunized in a parallel manner. Dosages effective to induce an immune response in humans are typically similar to dosages effective in mice.

III. SCREEN FOR THERAPEUTIC EFFICACY AGAINST ESTABLISHED AD

This assay is designed to test immunogenic agents for activity in arresting or reversing neuropathologic characteristics of AD in aged animals. Immunizations with 42 amino acid long Aβ (AN1792) were begun at a time point when amyloid plaques are already present in the brains of the PDAPP mice.

Over the time course used in this study, untreated PDAPP mice develop a number of neurodegenerative changes that resemble those found in AD (*Games et al., supra* and Johnson-Wood et al., *Proc. Natl. Acad. Sci. USA* 94, 1550-1555 (1997)). The deposition of Aβ into amyloid plaques is associated with a degenerative neuronal response consisting of aberrant axonal and dendritic elements, called dystrophic neurites. Amyloid deposits that are surrounded by and contain dystrophic neurites called neuritic plaques. In both AD and the PDAPP mouse, dystrophic neurites have a distinctive globular structure, are immunoreactive with a panel of antibodies recognizing APP and cytoskeletal components, and display complex subcellular degenerative changes at the ultrastructural level. These characteristics allow for disease-relevant, selective and reproducible measurements of neuritic plaque formation in the PDAPP brains. The dystrophic neuronal component of PDAPP neuritic plaques is easily visualized with an antibody specific for human APP (monoclonal antibody 8E5), and is readily measurable by computer-assisted image analysis. Therefore, in addition to measuring the effects of AN1792 on amyloid plaque formation, we monitored the effects of this treatment on the development of neuritic dystrophy.

Astrocytes and microglia are non-neuronal cells that respond to and reflect the degree of neuronal injury. GFAP-positive astrocytes and MHC II-positive microglia are commonly observed in AD, and their activation increases with the severity of the disease. Therefore, we also monitored the development of reactive astrocytosis and microgliosis in the AN1792-treated mice.

A. Materials and Methods

Forty-eight, heterozygous female PDAPP mice, 11 to 11.5 months of age, obtained from Charles River, were randomly divided into two groups: 24 mice to be immunized with 100 μg of AN1792 and 24 mice to be immunized with PBS, each combined with Freund's adjuvant. The AN1792 and PBS groups were again divided when they reached ~15 months of age. At 15 months of age approximately half of each group of the AN1792- and PBS-treated animals were euthanized (n=10 and 9, respectively), the remainder continued to receive immunizations until termination at ~18 months (n=9 and 12, respectively). A total of 8 animals (5 AN1792, 3 PBS) died during the study. In addition to the immunized animals, one-year old (n=10), 15-month old (n=10) and 18-month old (n=10) untreated PDAPP mice were included for comparison in the ELISAs to measure Aβ and APP levels in the brain; the one-year old animals were also included in the immunohistochemical analyses.

Methodology was as in Example 1 unless otherwise indicated. US Peptides lot 12 and California Peptides lot ME0339 of AN1792 were used to prepare the antigen for the six immunizations administered prior to the 15-month time point. California Peptides lots ME0339 and ME0439 were used for the three additional immunizations administered between 15 and 18 months.

For immunizations, 100 μg of AN1792 in 200 μl PBS or PBS alone was emulsified 1:1 (vol:vol) with Complete Freund's adjuvant (CFA) or Incomplete Freund's adjuvant (IFA) or PBS in a final volume of 400 μl. The first immunization was delivered with CFA as adjuvant, the next four doses were given with IFA and the final four doses with PBS alone without added adjuvant. A total of nine immunizations were given over the seven-month period on a two-week schedule for the first three doses followed by a four-week interval for the remaining injections. The four-month treatment group, euthanized at 15 months of age, received only the first 6 immunizations.

B. Results

1. Effects of AN1792 Treatment on Amyloid Burden

The results of AN1792 treatment on cortical amyloid burden determined by quantitative image analysis are shown in Fig. 7. The median value of cortical amyloid burden was 0.28% in a group of untreated 12-month old PDAPP mice, a value representative of the plaque load in mice at the study's initiation. At 18 months, the amyloid burden increased over 17-fold to 4.87% in PBS-treated mice, while AN1792-treated mice had a greatly reduced amyloid burden of only 0.01%, notably less than the 12-month untreated and both the 15- and 18-month PBS-treated groups. The amyloid burden was significantly reduced in the AN1792 recipients at both 15 (96% reduction; p=0.003) and 18 (>99% reduction; p=0.0002) months.

Typically, cortical amyloid deposition in PDAPP mice initiates in the frontal and retrosplenial cortices (RSC) and progresses in a ventral-lateral direction to involve the temporal and entorhinal cortices (EC). Little or no amyloid was found in the EC of 12 month-old mice, the approximate age at which AN1792 was first administered. After 4 months of AN1792 treatment, amyloid deposition was greatly diminished in the RSC, and the progressive involvement of the EC was entirely eliminated by AN1792 treatment. The latter observation showed that AN1792 completely halted the progression of amyloid that would normally invade the temporal and ventral cortices, as well as arrested or possibly reversed deposition in the RSC.

The profound effects of AN1792 treatment on developing cortical amyloid burden in the PDAPP mice are further demonstrated by the 18-month group, which had been treated for seven months. A near complete absence of cortical amyloid was found in the AN1792-treated mouse, with a total lack of diffuse plaques, as well as a reduction in compacted deposits.

2. AN1792 Treatment-associated Cellular and Morphological Changes

A population of Aβ-positive cells was found in brain regions that typically contain amyloid deposits. Remarkably, in several brains from AN1792 recipients, very few or no extracellular cortical amyloid plaques were found. Most of the Aβ immunoreactivity appeared to be contained within cells with large lobular or clumped soma. Phenotypically, these cells resembled activated microglia or monocytes. They were immunoreactive with antibodies recognizing ligands expressed by activated monocytes and microglia (MHC II and CD11b) and were occasionally associated with the wall or lumen of blood vessels. Comparison of near-adjacent sections labeled with Aβ and MHC II-specific antibodies revealed that similar patterns of these cells were recognized by both classes of antibodies. Detailed examination of the AN1792-treated brains revealed that the MHC II-positive cells were restricted to the vicinity of the limited amyloid remaining in these animals. Under the fixation conditions employed, the cells were not immunoreactive with antibodies that recognize T cell (CD3, CD3e) or B cell (CD45RA, CD45RB) ligands or leukocyte common antigen (CD45), but were reactive with an antibody recognizing leukosialin (CD43) which cross-reacts with monocytes. No such cells were found in any of the PBS-treated mice.

PDAPP mice invariably develop heavy amyloid deposition in the outer molecular layer of the hippocampal dentate gyrus. The deposition forms a distinct streak within the perforant pathway, a subregion that classically contains amyloid plaques in AD. The characteristic appearance of these deposits in PBS-treated mice resembled that previously characterized in untreated PDAPP mice. The amyloid deposition consisted of both diffuse and compacted plaques in a continuous band. In contrast, in a number of brains from AN1792-treated mice this pattern was drastically altered. The hippocampal amyloid deposition no longer contained diffuse amyloid, and the banded pattern was completely disrupted. Instead, a number of unusual punctate structures were present that are reactive with anti-Aβ antibodies, several of which appeared to be amyloid-containing cells.

MHC II-positive cells were frequently observed in the vicinity of extracellular amyloid in AN1792-treated animals. The pattern of association of Aβ-positive cells with amyloid was very similar in several brains from AN1792-treated mice. The distribution of these monocytic cells was restricted to the proximity of the deposited amyloid and was entirely absent from other brain regions devoid of Aβ plaques.

Confocal microscopy of MHCII- and Aβ-labelled sections revealed that plaque material was contained within many of the monocytic cells.

Quantitative image analysis of MHC II and MAC I-labeled sections revealed a trend towards increased immunoreactivity in the RSC and hippocampus of AN1792-treated mice compared to the PBS group which reached significance with the measure of MAC 1 reactivity in hippocampus.

These results are indicative of active, cell-mediated clearance of amyloid in plaque-bearing brain regions.

3.AN1792 Effects on Aβ Levels: ELISA Determinations

(a) Cortical Levels

In untreated PDAPP mice, the median level of total Aβ in the cortex at 12 months was 1,600 ng/g, which increased to 8,700 ng/g by 15 months (Table 2). At 18 months the value was 22,000 ng/g, an increase of over 10-fold during the time course of the experiment. PBS-treated animals had 8,600 ng/g total Aβ at 15 months which increased to 19,000 ng/g at 18 months. In contrast, AN1792-treated animals had 81% less total Aβ at 15 months (1,600 ng/g) than the PBS-immunized group. Significantly less (p=0.0001) total Aβ (5,200 ng/g) was found at 18 months when the AN1792 and PBS groups were compared (Table 2), representing a 72% reduction in the Aβ that would otherwise be present. Similar results were obtained when cortical levels of Aβ42 were compared, namely that the AN1792-treated group contained much less Aβ42, but in this case the differences between the AN1792 and PBS groups were significant at both 15 months (p=0.04) and 18 months (p=0.0001, Table 2).

Table 2: Median Aβ Levels (ng/g) in Cortex

	UNTREATED			PBS			AN1792		
Age	**Total**	**Aβ42**	**(n)**	**Total**	**Aβ42**	**(n)**	**Total**	**Aβ42**	**(n)**
12	1,600	1,300	(10)						
15	8,700	8,300	(10)	8,600	7,200	(9)	1,600	1,300*	(10)
18	22,200	18,500	(10)	19,000	15,900	(12)	5,200**	4,000**	(9)

* p = 0.0412
** p = 0.0001

(b) Hippocampal Levels

In untreated PDAPP mice, median hippocampal levels of total Aβ at twelve months of age were 15,000 ng/g which increased to 51,000 ng/g at 15 months and further to 81,000 ng/g at 18 months (Table 3). Similarly, PBS immunized mice showed values of 40,000 ng/g and 65,000 ng/g at 15 months and 18 months, respectively. AN1792 immunized animals exhibited less total Aβ, specifically 25,000 ng/g and 51,000 ng/g at the respective 15-month and 18-month timepoints. The 18-month AN1792-treated group value was significantly lower than that of the PBS treated group (p= 0.0105; Table 3). Measurement of Aβ42 gave the same pattern of results, namely that levels in the AN1792-treated group were significantly lower than in the PBS group (39,000 ng/g vs. 57,000 ng/g, respectively; p=0.002) at the 18-month evaluation (Table 3).

Table 3: Median Aβ Levels (ng/g) in Hippocampus

	UNTREATED			**PBS**			**AN1792**		
Age	Total	Aβ42	(n)	Total	Aβ42	(n)	Total	Aβ42	(n)
12	15,500	11,100	(10)						
15	51,500	44,400	(10)	40,100	35,70	(9)	24,50	22,100	(10)
18	80,800	64,200	(10)	65,400	57,10	(12)	50,90	38,900**	(9)

* p = 0.0105

** p = 0.0022

(c) Cerebellar Levels

In 12-month untreated PDAPP mice, the median cerebellar level of total Aβ was 15 ng/g (Table 4). At 15 months, this median increased to 28 ng/g and by 18 months had risen to 35 ng/g. PBS-treated animals displayed median total Aβ values of 21 ng/g at 15 months and 43 ng/g at 18 months. AN1792-treated animals were found to have 22 ng/g total Aβ at 15 months and significantly less (p=0.002) total Aβ at 18 months (25 ng/g) than the corresponding PBS group (Table 4).

Table 4: Median Aβ Levels (ng/g) in Cerebellum

	UNTREATED		**PBS**		**AN1792**	
Age	**Total Aβ**	**(n)**	**Total Aβ**	**(n)**	**Total Aβ**	**(n)**
12	15.6	(10)				
15	27.7	(10)	20.8	(9)	21.7	(10)
18	35.0	(10)	43.1	(12)	24.8*	(9)

* p = 0.0018

4. Effects of AN1792 Treatment on APP Levels

APP-α and the full-length APP molecule both contain all or part of the Aβ sequence and thus could be potentially impacted by the generation of an AN1792-directed immune response. In studies to date, a slight increase in APP levels has been noted as neuropathology increases in the PDAPP mouse. In the cortex, levels of either APP-α /FL (full length) or APP-α were essentially unchanged by treatment with the exception that APP-α was reduced by 19% at the 18-month timepoint in the AN1792-treated vs. the PBS-treated group. The 18-month AN1792-treated APP values were not significantly different from values of the 12-month and 15-month untreated and 15-month PBS groups. In all cases the APP values remained within the ranges that are normally found in PDAPP mice.

5. Effects of AN1792 Treatment on Neurodegenerative and Gliotic Pathology

Neuritic plaque burden was significantly reduced in the frontal cortex of AN1792-treated mice compared to the PBS group at both 15 (84%; p=0.03) and 18 (55%; p=0.01) months of age (Fig. 8). The median value of the neuritic plaque burden increased from 0.32% to 0.49% in the PBS group between 15 and 18 months of age. This contrasted with the greatly reduced development of neuritic plaques in the AN1792 group, with median neuritic plaque burden values of 0.05% and 0.22%, in the 15 and 18 month groups, respectively.

Immunizations with AN1792 seemed well tolerated and reactive astrocytosis was also significantly reduced in the RSC of AN1792-treated mice when compared to the PBS group at both 15 (56%; p=0.011) and 18 (39%; p=0.028) months of age (Fig. 9). Median values of the percent of astrocytosis in the PBS group increased between 15 and 18 months from 4.26% to 5.21%. AN1792-treatment suppressed the development of astrocytosis at both time points to 1.89% and 3.2%, respectively. This suggests the neuropil was not being damaged by the clearance process.

6. Antibody Responses

As described above, eleven-month old, heterozygous PDAPP mice (N=24) received a series of 5 immunizations of 100 μg of AN1792 emulsified with Freund's adjuvant and administered intraperitoneally at weeks 0, 2, 4, 8, and 12, and a sixth immunization with PBS alone (no Freund's adjuvant) at week 16. As a negative control, a parallel set of 24 age-matched transgenic mice received immunizations of PBS emulsified with the same adjuvants and delivered on the same schedule. Animals were bled within three to seven days following each immunization starting after the second dose. Antibody responses to AN1792 were measured by ELISA. Geometric mean titers (GMT) for the animals that were immunized with AN1792 were approximately 1,900, 7,600, and 45,000 following the second, third and last (sixth) doses respectively. No Aβ-specific antibody was measured in control animals following the sixth immunization.

Approximately one-half of the animals were treated for an additional three months, receiving immunizations at about 20, 24 and 27 weeks. Each of these doses was delivered in PBS vehicle alone without Freund's adjuvant. Mean antibody titers remained unchanged over this time period. In fact, antibody titers appeared to remain stable from the fourth to the eighth bleed corresponding to a period covering the fifth to the ninth injections.

To determine if the Aβ-specific antibodies elicited by immunization that were detected in the sera of AN1792-treated mice were also associated with deposited brain amyloid, a subset of sections from the AN1792- and PBS-treated mice were reacted with an antibody specific for mouse IgG. In contrast to the PBS group, Aβ plaques in AN1792-treated brains were coated with endogenous IgG. This difference between the two groups was seen in both 15-and 18-month groups. Particularly striking was the lack of labeling in the PBS group, despite the presence of a heavy amyloid burden in these mice. These results show that immunization with a synthetic Aβ protein generates antibodies that recognize and bind in vivo to the Aβ in amyloid plaques.

7. Cellular-Mediated Immune Responses

Spleens were removed from nine AN1792-immunized and 12 PBS-immunized 18-month old PDAPP mice 7 days after the ninth immunization. Splenocytes were isolated and cultured for 72 h in the presence of Aβ40, Aβ42, or Aβ40-1 (reverse order protein). The mitogen Con A served as a positive control. Optimum responses were obtained with >1.7 μM protein. Cells from all nine AN1792-treated animals proliferated in response to either Aβ1-40 or Aβ1-42 protein, with equal levels of incorporation for both proteins (Fig. 10, Upper Panel). There was no response to the Aβ40-1 reverse protein. Cells from control animals did not respond to any of the Aβ proteins (Fig. 10, Lower Panel).

C. Conclusion

The results of this study show that AN1792 immunization of PDAPP mice possessing existing amyloid deposits slows and prevents progressive amyloid deposition and retard consequential neuropathologic changes in the aged PDAPP mouse brain. Immunizations with AN1792 essentially halted amyloid developing in structures that would normally succumb to amyloidosis. Thus, administration of Aβ peptide has therapeutic benefit in the treatment of AD.

IV. SCREEN OF Aβ FRAGMENTS

100 PDAPP mice age 9-11 months were immunized with 9 different regions of APP and Aβ to determine which epitopes convey the efficacious response. The 9 different immunogens and one control are injected i.p. as described above. The immunogens include four human Aβ peptide conjugates 1-12, 13-28, 32-42, 1-5, all coupled to sheep anti-mouse IgG via a cystine link; an APP polypeptide amino acids 592-695, aggregated human Aβ 1-40, and aggregated human Aβ 25-35, and aggregated rodent Aβ42. Aggregated Aβ42 and PBS were used as positive and negative controls, respectively. Ten mice were used per treatment group. Titers were monitored as above and mice were euthanized at the end of 4 months of injections. Histochemistry, Aβ levels, and toxicology analysis was determined post mortem.

A. Materials and Methods

1. Preparation of Immunogens

Preparation of coupled Aβ peptides: four human Aβ peptide conjugates (amino acid residues 1-5, 1-12, 13-28, and 33-42, each conjugated to sheep anti-mouse IgG) were prepared by coupling through an artificial cysteine added to the Aβ peptide using the crosslinking reagent sulfo-EMCS. The Aβ peptide derivatives were synthesized with the following final amino acid sequences. In each case, the location of the inserted cysteine residue is indicated by underlining. The Aβ13-28 peptide derivative also had two glycine residues added prior to the carboxyl terminal cysteine as indicated.

Aβ1-12 peptide	NH2-DAEFRHDSGYEVC-COOH
Aβ1-5 peptide	NH2-DAEFRC-COOH
Aβ33-42 peptide	NH2-C-amino-heptanoic acid-GLMVGGVVIA-COOH
Aβ13-28 peptide	Ac-NH-HHQKLVFFAEDVGSNKGGC-COOH

To prepare for the coupling reaction, ten mg of sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories) was dialyzed overnight against 10 mM sodium borate buffer, pH 8.5. The dialyzed antibody was then concentrated to a volume of 2 mL using an Amicon Centriprep tube. Ten mg sulfo-EMCS [N (ε-maleimidocuproyloxy) succinimide] (Molecular Sciences Co.) was dissolved in one mL deionized water. A 40-fold molar excess of sulfo-EMCS was added dropwise with stirring to the sheep anti-mouse IgG and then the solution was stirred for an additional ten min. The activated sheep anti-mouse IgG was purified and buffer exchanged by passage over a 10 mL gel filtration column (Pierce Presto Column, obtained from Pierce Chemicals) equilibrated with 0.1 M NaPO4, 5 mM EDTA, pH 6.5. Antibody containing fractions, identified by absorbance at 280 nm, were pooled and diluted to a concentration of approximately 1 mg/mL, using 1.4 mg per OD as the extinction coefficient. A 40-fold molar excess of Aβ peptide was dissolved in 20 mL of 10 mM NaPO4, pH 8.0, with the exception of the Aβ33-42 peptide for which 10 mg was first dissolved in 0.5 mL of DMSO and then diluted to 20 mL with the 10 mM NaPO4 buffer. The peptide solutions were each added to 10 mL of activated sheep anti-mouse IgG and rocked at room temperature for 4 hr. The resulting conjugates were concentrated to a final volume of less than 10 mL using an Amicon Centriprep tube and then dialyzed against PBS to buffer exchange the buffer and remove free peptide. The conjugates were passed through 0.22 μm-pore size filters for sterilization and then aliquoted into fractions of 1 mg and stored frozen at -20°C. The concentrations of the conjugates were determined using the BCA protein assay (Pierce Chemicals) with horse IgG for the standard curve. Conjugation was documented by the molecular weight increase of the conjugated peptides relative to that of the activated sheep anti-mouse IgG. The Aβ 1-5 sheep anti-mouse conjugate was a pool of two conjugations, the rest were from a single preparation.

2. Preparation of aggregated Aβ peptides

Human 1-40 (AN1528; California Peptides Inc., Lot ME0541), human 1-42 (AN1792; California Peptides Inc., Lots ME0339 and ME0439), human 25-35, and rodent 1-42 (California Peptides Inc., Lot ME0218) peptides were freshly solubilized for the preparation of each set of injections from lyophilized powders that had been stored desiccated at -20°C. For this purpose, two mg of peptide were added to 0.9 ml of deionized water and the mixture was vortexed to generate a relatively uniform solution or suspension. Of the four, AN1528 was the only peptide soluble at this step. A 100 μl aliquot of 10X PBS (1X PBS: 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was then added at which point AN1528 began to precipitate. The suspension was vortexed again and incubated overnight at 37°C for use the next day.

Preparation of the pBx6 protein: An expression plasmid encoding pBx6, a fusion protein consisting of the 100-amino acid bacteriophage MS-2 polymerase N-terminal leader sequence followed by amino acids 592-695 of APP (βAPP) was constructed as described by Oltersdorf et al., J. Biol. Chem. 265, 4492-4497 (1990). The plasmid was transfected into E. coli and the protein was expressed after induction of the promoter. The bacteria were lysed in 8M urea and pBx6 was partially purified by preparative SDS PAGE. Fractions containing pBx6 were identified by Western blot using a rabbit anti-pBx6 polyclonal antibody, pooled, concentrated using an Amicon Centriprep tube and dialysed against PBS. The purity of the preparation, estimated by Coomassie Blue stained SDS PAGE, was approximately 5 to 10%.

B. Results and Discussion

1. Study Design

One hundred male and female, nine- to eleven-month old heterozygous PDAPP transgenic mice were obtained from Charles River Laboratory and Taconic Laboratory. The mice were sorted into ten groups to be immunized with different regions of Aβ or APP combined with Freund's adjuvant. Animals were distributed to match the gender, age, parentage and source of the animals within the groups as closely as possible. The immunogens included four Aβ peptides derived from the human sequence, 1-5, 1-12, 13-28, and 33-42, each conjugated to sheep anti-mouse IgG; four aggregated Aβ peptides, human 1-40 (AN1528), human 1-42 (AN1792), human 25-35, and rodent 1-42; and a fusion polypeptide, designated as pBx6, containing APP amino acid residues 592-695. A tenth group was immunized with PBS combined with adjuvant as a control.

For each immunization, 100 μg of each Aβ peptide in 200 μl PBS or 200 μg of the APP derivative pBx6 in the same volume of PBS or PBS alone was emulsified 1:1 (vol:vol) with Complete Freund's adjuvant (CFA) in a final volume of 400 μl for the first immunization, followed by a boost of the same amount of immunogen in Incomplete Freund's adjuvant (IFA) for the subsequent four doses and with PBS for the final dose. Immunizations were delivered intraperitoneally on a biweekly schedule for the first three doses, then on a monthly schedule thereafter. Animals were bled four to seven days following each immunization starting after the second dose for the measurement of antibody titers. Animals were euthanized approximately one week after the final dose.

2. Aβ and APP Levels in the Brain

Following about four months of immunization with the various Aβ peptides or the APP derivative, brains were removed from saline-perfused animals. One hemisphere was prepared for immunohistochemical analysis and the second was used for the quantitation of Aβ and APP levels. To measure the concentrations of various forms of beta amyloid peptide and amyloid precursor protein, the hemisphere was dissected and homogenates of the hippocampal, cortical, and cerebellar regions were prepared in 5 M guanidine. These were diluted and the level of amyloid or APP was quantitated by comparison to a series of dilutions of standards of Aβ peptide or APP of known concentrations in an ELISA format.

The median concentration of total Aβ for the control group immunized with PBS was 5.8-fold higher in the hippocampus than in the cortex (median of 24,318 ng/g hippocampal tissue compared to 4,221 ng/g for the cortex). The median level in the cerebellum of the control group (23.4 ng/g tissue) was about 1,000-fold lower than in the hippocampus. These levels are similar to those that we have previously reported for heterozygous PDAPP transgenic mice of this age (Johnson-Woods et al., 1997, supra).

For the cortex, a subset of treatment groups had median total Aβ and Aβ1-42 levels which differed significantly from those of the control group ($p < 0.05$), those animals receiving AN1792, rodent Aβ1-42 or the Aβ1-5 peptide conjugate as shown in Fig. 11. The median levels of total Aβ were reduced by 75%, 79% and 61%, respectively, compared to the control for these treatment groups. There were no discernable correlations between Aβ-specific antibody titers and Aβ levels in the cortical region of the brain for any of the groups.

In the hippocampus, the median reduction of total Aβ associated with AN1792 treatment (46%, p = 0.0543) was not as great as that observed in the cortex (75%, p = 0.0021). However, the magnitude of the reduction was far greater in the hippocampus than in the cortex, a net reduction of 11,186 ng/g tissue in the hippocampus versus 3,171 ng/g tissue in the cortex. For groups of animals receiving rodent Aβ1-42 or Aβ1-5, the median total Aβ levels were reduced by 36% and 26%, respectively. However, given the small group sizes and the high variability of the amyloid peptide levels from animal to animal within both groups, these reductions were not significant. When the levels of Aβ1-42 were measured in the hippocampus, none of the treatment-induced reductions reached significance. Thus, due to the smaller Aβ burden in the cortex, changes in this region are a more sensitive indicator of treatment effects. The changes in Aβ levels measured by ELISA in the cortex are similar, but not identical, to the results from the immunohistochemical analysis (see below).

Total Aβ was also measured in the cerebellum, a region typically minimally affected with AD pathology. None of the median Aβ concentrations of any of the groups immunized with the various Aβ peptides or the APP derivative differed from that of the control group in this region of the brain. This result suggests that non-pathological levels of Aβ are unaffected by treatment.

APP concentration was also determined by ELISA in the cortex and cerebellum from treated and control mice. Two different APP assays were utilized. The first, designated APP-α /FL, recognizes both APP-alpha (α, the secreted form of APP which has been cleaved within the Aβ sequence), and full-length forms (FL) of APP, while the second recognizes only APP-α. In contrast to the treatment-associated diminution of Aβ in a subset of treatment groups, the levels of APP were unchanged in all of the treated compared to the control animals. These results indicate that the immunizations with Aβ peptides are not depleting APP; rather the treatment effect is specific to Aβ.

In summary, total Aβ and Aβ1-42 levels were significantly reduced in the cortex by treatment with AN1792, rodent Aβ1-42 or Aβ1-5 conjugate. In the hippocampus, total Aβ was significantly reduced only by AN1792 treatment. No other treatment-associated changes in Aβ or APP levels in the hippocampal, cortical or cerebellar regions were significant.

2. Histochemical Analyses

Brains from a subset of six groups were prepared for immunohistochemical analysis, three groups immunized with the Aβ peptide conjugates Aβ1-5, Aβ1-12, and Aβ13-28; two groups immunized with the full length Aβ aggregates AN1792 and AN1528 and the PBS-treated control group. The results of image analyses of the amyloid burden in brain sections from these groups are shown in Fig. 12. There were significant reductions of amyloid burden in the cortical regions of three of the treatment groups versus control animals. The greatest reduction of amyloid burden was observed in the group receiving AN1792 where the mean value was reduced by 97% ($p = 0.001$). Significant reductions were also observed for those animals treated with AN1528 (95%, $p = 0.005$) and the Aβ1-5 peptide conjugate (67%, $p = 0.02$).

The results obtained by quantitation of total Aβ or Aβ1-42 by ELISA and amyloid burden by image analysis differ to some extent. Treatment with AN1528 had a significant impact on the level of cortical amyloid burden when measured by quantitative image analysis but not on the concentration of total Aβ in the same region when measured by ELISA. The difference between these two results is likely to be due to the specificities of the assays. Image analysis measures only insoluble Aβ aggregated into plaques. In contrast, the ELISA measures all forms of Aβ, both soluble and insoluble, monomeric and aggregated. Since the disease pathology is thought to be associated with the insoluble plaque-associated form of Aβ, the image analysis technique may have more sensitivity to reveal treatment effects. However since the ELISA is a more rapid and easier assay, it is very useful for screening purposes. Moreover it may reveal that the treatment-associated reduction of Aβ is greater for plaque-associated than total Aβ.

To determine if the Aβ-specific antibodies elicited by immunization in the treated animals reacted with deposited brain amyloid, a subset of the sections from the treated animals and the control mice were reacted with an antibody specific for mouse IgG. In contrast to the PBS group, Aβ-containing plaques were coated with endogenous IgG for animals immunized with the Aβ peptide conjugates Aβ1-5, Aβ1-12, and Aβ13-28; and the full length Aβ aggregates AN1792 and AN1528. Brains from animals immunized with the other Aβ peptides or the APP peptide pBx6 were not analyzed by this assay.

3. Measurement of Antibody Titers

Mice were bled four to seven days following each immunization starting after the second immunization, for a total of five bleeds. Antibody titers were measured as Aβ1-42-binding antibody using a sandwich ELISA with plastic multi-well plates coated with Aβ1-42. As shown in Fig. 13, peak antibody titers were elicited following the fourth dose for those four immunogenic formulations which elicited the highest titers of AN1792-specific antibodies: AN1792 (peak GMT: 94,647), AN1528 (peak GMT: 88,231), Aβ1-12 conjugate (peak GMT: 47,216)and rodent Aβ1-42 (peak GMT: 10,766). Titers for these groups declined somewhat following the fifth and sixth doses. For the remaining five immunogens, peak titers were reached following the fifth or the sixth dose and these were of much lower magnitude than those of the four highest titer groups: Aβ1-5 conjugate (peak GMT: 2,356), pBx6 (peak GMT: 1,986), Aβ13-28 conjugate (peak GMT: 1,183), Aβ33-42 conjugate (peak GMT: 658), Aβ25-35 (peak GMT: 125). Antibody titers were also measured against the homologous peptides using the same ELISA sandwich format for a subset of the immunogens, those groups immunized with Aβ1-5, Aβ13-28, Aβ25-35, Aβ33-42 or rodent Aβ1-42. These titers were about the same as those measured against Aβ1-42 except for the rodent Aβ1-42 immunogen in which case antibody titers against the homologous immunogen were about two-fold higher. The magnitude of the AN1792-specific antibody titer of individual animals or the mean values of treatment groups did not correlate with efficacy measured as the reduction of Aβ in the cortex.

4. Lymphoproliferative Responses

Aβ-dependent lymphoproliferation was measured using spleen cells harvested approximately one week following the final, sixth, immunization. Freshly harvested cells, 105 per well, were cultured for 5 days in the presence of Aβ1-40 at a concentration of 5 μM for stimulation. Cells from a subset of seven of the ten groups were also cultured in the presence of the reverse peptide, Aβ40-1. As a positive control, additional cells were cultured with the T cell mitogen, PHA, and, as a negative control, cells were cultured without added peptide.

Lymphocytes from a majority of the animals proliferated in response to PHA. There were no significant responses to the Aβ40-1 reverse peptide. Cells from animals immunized with the larger aggregated Aβ peptides, AN1792, rodent Aβ1-42 and AN1528 proliferated robustly when stimulated with Aβ1-40 with the highest cpm in the recipients of AN1792. One animal in each of the groups immunized with Aβ1-12 conjugate, Aβ13-28 conjugate and Aβ25-35 proliferated in response to Aβ1-40. The remaining groups receiving Aβ1-5 conjugate, Aβ33-42 conjugate pBx6 or PBS had no animals with an Aβ-stimulated response. These results are summarized in Table 5 below.

Table 5

Immunogen	Conjugate	Aβ Amino Acids	Responders
Aβ1-5	Yes	5-mer	0/7
Aβ1-12	Yes	12-mer	1/8
Aβ13-28	Yes	16-mer	1/9
Aβ25-35		11-mer	1/9
Aβ33-42	Yes	10-mer	0/10
Aβ1-40		40-mer	5/8
Aβ1-42		42-mer	9/9
r Aβ1-42		42-mer	8/8
pBx6			0/8
PBS		0-mer	0/8

These results show that AN1792 and AN1528 stimulate strong T cell responses, most likely of the CD4+ phenotype. The absence of an Aβ-specific T cell response in animals immunized with Aβ1-5 is not surprising since peptide epitopes recognized by CD4+ T cells are usually about 15 amino acids in length, although shorter peptides can sometimes function with less efficiency. Thus the majority of helper T cell epitopes for the four conjugate peptides are likely to reside in the IgG conjugate partner, not in the Aβ region. This hypothesis is supported by the very low incidence of proliferative responses for animals in each of these treatment groups. Since the Aβ1-5 conjugate was effective at significantly reducing the level of Aβ in the brain, in the apparent absence of Aβ-specific T cells, the key effector immune response induced by immunization with this peptide appears to be antibody.

Lack of T-cell and low antibody response from fusion peptide pBx6, encompassing APP amino acids 592-695 including all of the Aβ residues may be due to the poor immunogenicity of this particular preparation. The poor immunogenicity of the Aβ25-35 aggregate is likely due to the peptide being too small to be likely to contain a good T cell epitope to help the induction of an antibody response. If this peptide were conjugated to a carrier protein, it would probably be more immunogenic.

V. Preparation of Polyclonal Antibodies for Passive Protection

125 non-transgenic mice were immunized with 100 μg Aβ1-42, plus CFA/IFA adjuvant, and euthanized at 4-5 months. Blood was collected from immunized mice. IgG was separated from other blood components. Antibody specific for the immunogen may be partially purified by affinity chromatography. An average of about 0.5-1 mg of immunogen-specific antibody is obtained per mouse, giving a total of 60-120 mg.

VI. Passive Immunization with Antibodies to Aβ

Groups of 7-9 month old PDAPP mice each were injected with 0.5 mg in PBS of polyclonal anti-Aβ or specific anti-Aβ monoclonals as shown below. All antibody preparations were purified to have low endotoxin levels. Monoclonals can be prepared against a fragment by injecting the fragment or longer form of Aβ into a mouse, preparing hybridomas and screening the hybridomas for an antibody that specifically binds to a desired fragment of Aβ without binding to other nonoverlapping fragments of Aβ.

Table 6

Antibody	Epitope
2H3	Aβ 1-12
10D5	Aβ 1-12
266	Aβ 13-28
21F12	Aβ 33-42
Mouse polyclonal anti-human Aβ42	Anti-Aggregated Aβ42

Mice were injected ip as needed over a 4 month period to maintain a circulating antibody concentration measured by ELISA titer of greater than 1/1000 defined by ELISA to Aβ42 or other immunogen. Titers were monitored as above and mice were euthanized at the end of 6 months of injections. Histochemistry, Aβ levels and toxicology were performed post mortem. Ten mice were used per group. Additional studies of passive immunization are described in Examples XI and XII below.

VII. Comparison of Different Adjuvants

This example compares CFA, alum, an oil-in water emulsion and MPL for capacity to stimulate an immune response.

A. Materials and Methods

1. Study Design

One hundred female Hartley strain six-week old guinea pigs, obtained from Elm Hill, were sorted into ten groups to be immunized with AN1792 or a palmitoylated derivative thereof combined with various adjuvants. Seven groups received injections of AN1792 (33 μg unless otherwise specified) combined with a) PBS, b) Freund's adjuvant, c) MPL, d) squalene, e) MPL/squalene f) low dose alum, or g) high dose alum (300 μg AN1792). Two groups received injections of a palmitoylated derivative of AN1792 (33 μg) combined with a) PBS or b) squalene. A final, tenth group received PBS alone without antigen or additional adjuvant. For the group receiving

Freund's adjuvant, the first dose was emulsified with CFA and the remaining four doses with IFA. Antigen was administered at a dose of 33 μg for all groups except the high dose alum group, which received 300 μg of AN1792. Injections were administered intraperitoneally for CFA/IFA and intramuscularly in the hind limb quadriceps alternately on the right and left side for all other groups. The first three doses were given on a biweekly schedule followed by two doses at a monthly interval). Blood was drawn six to seven days following each immunization, starting after the second dose, for measurement of antibody titers.

2. Preparation of Immunogens

Two mg Aβ42 (California Peptide, Lot ME0339) was added to 0.9 ml of deionized water and the mixture was vortexed to generate a relatively uniform suspension. A 100 μl aliquot of 10X PBS (1X PBS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was added. The suspension was vortexed again and incubated overnight at 37°C for use the next day. Unused Aβ1-42 was stored with desiccant as a lyophilized powder at -20°C.

A palmitoylated derivative of AN1792 was prepared by coupling palmitic anhydride, dissolved in dimethyl formamide, to the amino terminal residue of AN1792 prior to removal of the nascent peptide from the resin by treatment with hydrofluoric acid.

To prepare formulation doses with Complete Freund's adjuvant (CFA) (group 2), 33 μg of AN1792 in 200 μl PBS was emulsified 1:1 (vol:vol) with CFA in a final volume of 400 μl for the first immunization. For subsequent immunizations, the antigen was similarly emulsified with Incomplete Freund's adjuvant (IFA).

To prepare formulation doses with MPL for groups 5 and 8, lyophilized powder (Ribi ImmunoChem Research, Inc., Hamilton, MT) was added to 0.2% aqueous triethylamine to a final concentration of 1 mg/ml and vortexed. The mixture was heated to 65 to 70°C for 30 sec to create a slightly opaque uniform suspension of micelles. The solution was freshly prepared for each set of injections. For each injection in group 5, 33 μg of AN1792 in 16.5 μl PBS, 50 μg of MPL (50 μl) and 162 μl of PBS were mixed in a borosilicate tube immediately before use.

To prepare formulation doses with the low oil-in-water emulsion, AN1792 in PBS was added to 5% squalene, 0.5% Tween 80, 0.5% Span 85 in PBS to reach a final single dose concentration of 33 μg AN1792 in 250 μl (group 6). The mixture was emulsified by passing through a two-chambered hand-held device 15 to 20 times until the emulsion droplets appeared to be about equal in diameter to a 1.0 μm diameter standard latex bead when viewed under a microscope. The resulting suspension was opalescent, milky white. The emulsions were freshly prepared for each series of injections. For group 8, MPL in 0.2% triethylamine was added at a concentration of 50 μg per dose to the squalene and detergent mixture for emulsification as noted above. For the palmitoyl derivative (group 7), 33 μg per dose of palmitoyl-NH-Aβ1-42 was added to squalene and vortexed. Tween 80 and Span 85 were then added with vortexing. This mixture was added to PBS to reach final concentrations of 5% squalene, 0.5% Tween 80, 0.5% Span 85 and the mixture was emulsified as noted above.

To prepare formulation doses with alum (groups 9 and 10), AN1792 in PBS was added to Alhydrogel (aluminum hydroxide gel, Accurate, Westbury, NY) to reach concentrations of 33 μg (low dose, group 9) or 300 μg (high dose, group 10) AN1792 per 5 mg of alum in a final dose volume of 250 μl. The suspension was gently mixed for 4 hr at RT.

3. Measurement of Antibody Titers

Guinea pigs were bled six to seven days following immunization starting after the second immunization for a total of four bleeds. Antibody titers against Aβ42 were measured by ELISA as described in General Materials and Methods.

4. Tissue Preparation

After about 14 weeks, all guinea pigs were euthanized by administering CO_2. Cerebrospinal fluid was collected and the brains were removed and three brain regions (hippocampus, cortex and cerebellum) were dissected and used to measure the concentration of total Aβ protein using ELISA.

B. Results

1. Antibody Responses

There was a wide range in the potency of the various adjuvants when measured as the antibody response to AN1792 following immunization. As shown in Fig. 14, when AN1792 was administered in PBS, no antibody was detected following two or three immunizations and negligible responses were detected following the fourth and fifth doses with geometric mean titers (GMTs) of only about 45. The o/w emulsion induced modest titers following the third dose (GMT 255) that were maintained following the fourth dose (GMT 301) and fell with the final dose (GMT 54). There was a clear antigen dose response for AN1792 bound to alum with 300 μg being more immunogenic at all time points than 33 μg. At the peak of the antibody response, following the fourth immunization, the difference between the two doses was 43% with GMTs of about 1940 (33 μg) and 3400 (300 μg). The antibody response to 33 μg AN1792 plus MPL was very similar to that generated with almost a ten-fold higher dose of antigen (300 μg) bound to alum. The addition of MPL to an o/w emulsion decreased the potency of the formulations relative to that with MPL as the sole adjuvant by as much as 75%. A palmitoylated derivative of AN1792 was completely non-immunogenic when administered in PBS and gave modest titers when presented in an o/w emulsion with GMTs of 340 and 105 for the third and fourth bleeds. The highest antibody titers were generated with Freund's adjuvant with a peak GMT of about 87,000, a value almost 30-fold greater than the GMTs of the next two most potent formulations, MPL and high dose AN1792/alum.

The most promising adjuvants identified in this study are MPL and alum. Of these two, MPL appears preferable because a 10-fold lower antigen dose was required to generate the same antibody response as obtained with alum. The response can be increased by increasing the dose of antigen and /or adjuvant and by optimizing the immunization schedule. The o/w emulsion was a very weak adjuvant for AN1792 and adding an o/w emulsion to MPL adjuvant diminished the intrinsic adjuvant activity of MPL alone.

2. Aβ Levels In The Brain

At about 14 weeks the guinea pigs were deeply anesthetized, the cerebrospinal fluid (CSF) was drawn and brains were excised from animals in a subset of the groups, those immunized with Freund's adjuvant (group 2), MPL (group 5), alum with a high dose, 300 μg, of AN1792 (group 10) and the PBS immunized control group (group 3). To measure the level of Aβ peptide, one hemisphere was dissected and homogenates of the hippocampal, cortical, and cerebellar regions were prepared in 5 M guanidine. These were diluted and quantitated by comparison to a series of dilutions of Aβ standard protein of known concentrations in an ELISA format. The levels of Aβ protein in the hippocampus, the cortex and the cerebellum were very similar for all four groups despite the wide range of antibody responses to Aβ elicited by these formulations. Mean Aβ levels of about 25 ng/g tissue were measured in the hippocampus, 21 ng/g in the cortex, and 12 ng/g in the cerebellum. Thus, the presence of a high circulating antibody titer to Aβ for almost three months in some of these animals did not alter the total Aβ levels in their brains. The levels of Aβ in the CSF were also quite similar between the groups. The lack of large effect of AN1792 immunization on endogenous Aβ indicates that the immune response is focused on pathological formations of Aβ.

VIII. Immune Response to Different Adjuvants in Mice

Six-week old female Swiss Webster mice were used for this study with 10-13 animals per group. Immunizations were given on days 0, 14, 28, 60, 90 and 20 administered subcutaneously in a dose volume of 200 μl. PBS was used as the buffer for all formulations. Animals were bleed seven days following each immunization starting after the second dose for analysis of antibody titers by ELISA. The treatment regime of each group is summarized in Table 7.

Table 7

Experimental Design					
Group	N[a]	Adjuvant[b]	Dose	Antigen	Dose (μg)
1	10	MPL	12.5 μg	AN1792	33
2	10	MPL	25 μg	AN1792	33
3	10	MPL	50 μg	AN1792	33
4	13	MPL	125 μg	AN1792	33
5	13	MPL	50 μg	AN1792	150
6	13	MPL	50 μg	AN1528	33
7	10	PBS		AN1792	33
8	10	PBS		None	
9	10	Squalene emulsified	5%	AN1792	33
10	10	Squalene admixed	5%	AN1792	33
11	10	Alum	2 mg	AN1792	33
12	13	MPL + Alum	50 μg/2 mg	AN1792	33
13	10	QS-21	5 μg	AN1792	33
14	10	QS-21	10 μg	AN1792	33
15	10	QS-21	25 AN1792	AN1792	33
16	13	QS-21	25 AN1792	AN1792	150
17	13	QS-21	25 AN1792	AN1528	33
18	13	QS-21 + MPL	25 μg/50 μg	AN1792	33
19	13	QS-21 + Alum	25 μg/2 mg	AN1792	33

Footnotes:

[a] Number of mice in each group at the initiation of the experiment.

[b] The adjuvants are noted. The buffer for all these formulations was PBS. For group 8, there was no adjuvant and no antigen.

The ELISA titers of antibodies against Aβ42 in each group are shown in Table 8 below.

Table 8.

Geometric Mean Antibody Titers Week of Bleed					
Treatment Group	2.9	5.0	8.7	12.9	16.7
1	248	1797	2577	6180	4177
2	598	3114	3984	5287	6878
3	1372	5000	7159	12333	12781
4	1278	20791	14368	20097	25631
5	3288	26242	13229	9315	23742
6	61	2536	2301	1442	4504
7	37	395	484	972	2149
8	25	25	25	25	25
9	25	183	744	952	1823
10	25	89	311	513	817
11	29	708	2618	2165	3666
12	198	1458	1079	612	797
13	38	433	566	1080	626
14	104	541	3247	1609	838
15	212	2630	2472	1224	1496
16	183	2616	6680	2085	1631
17	28	201	375	222	1540
18	31699	15544	23095	6412	9059
19	63	243	554	299	441

The table shows that the highest titers were obtained for groups 4, 5 and 18, in which the adjuvants were 125 µg MPL, 50 µg MPL and QS-21 plus MPL.

IX. Therapeutic Efficacy of Different Adjuvants

A therapeutic efficacy study was conducted in PDAPP transgenic mice with a set of adjuvants suitable for use in humans to determine their ability to potentiate immune responses to Aβ and to induce the immune-mediated clearance of amyloid deposits in the brain.

One hundred eighty male and female, 7.5- to 8.5-month old heterozygous PDAPP transgenic mice were obtained from Charles River Laboratories. The mice were sorted into nine groups containing 15 to 23 animals per group to be immunized with

AN1792 or AN1528 combined with various adjuvants. Animals were distributed to match the gender, age, and parentage of the animals within the groups as closely as possible. The adjuvants included alum, MPL, and QS-21, each combined with both antigens, and Freund's adjuvant (FA) combined with only AN1792. An additional group was immunized with AN1792 formulated in PBS buffer plus the preservative thimerosal without adjuvant. A ninth group was immunized with PBS alone as a negative control.

Preparation of aggregated Aβ peptides: human Aβ1-40 (AN1528; California Peptides Inc., Napa, CA; Lot ME0541) and human Aβ1-42 (AN1792; California Peptides Inc., Lot ME0439) peptides were freshly solubilized for the preparation of each set of injections from lyophilized powders that had been stored desiccated at -20°C. For this purpose, two mg of peptide were added to 0.9 ml of deionized water and the mixture was vortexed to generate a relatively uniform solution or suspension. AN1528 was soluble at this step, in contrast to AN1792. A 100 μl aliquot of 10X PBS (1X PBS: 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was then added at which point AN1528 began to precipitate. The suspensions were vortexed again and incubated overnight at 37°C for use the next day.

To prepare formulation doses with alum (Groups 1 and 5), Aβ peptide in PBS was added to Alhydrogel (two percent aqueous aluminum hydroxide gel, Sargeant, Inc., Clifton, NJ) to reach concentrations of 100 μg Aβ peptide per 1 mg of alum. 10X PBS was added to a final dose volume of 200 μl in 1X PBS. The suspension was then gently mixed for approximately 4 hr at RT prior to injection.

To prepare formulation doses for with MPL (Groups 2 and 6), lyophilized powder (Ribi ImmunoChem Research, Inc., Hamilton, MT; Lot 67039-E0896B) was added to 0.2% aqueous triethylamine to a final concentration of 1 mg/ml and vortexed. The mixture was heated to 65 to 70°C for 30 sec to create a slightly opaque uniform suspension of micelles. The solution was stored at 4°C. For each set of injections, 100 μg of peptide per dose in 50 μl PBS, 50 μg of MPL per dose (50 μl) and 100 μl of PBS per dose were mixed in a borosilicate tube immediately before use.

To prepare formulation doses with QS-21 (Groups 3 and 7), lyophilized powder (Aquila, Framingham, MA; Lot A7018R) was added to PBS, pH 6.6-6.7 to a final concentration of 1 mg/ml and vortexed. The solution was stored at -20°C. For each set of injections, 100 μg of peptide per dose in 50 μl PBS, 25 μg of QS-21 per dose in 25 μl PBS and 125 μl of PBS per dose were mixed in a borosilicate tube immediately before use.

To prepare formulation doses with Freund's Adjuvant (Group 4), 100 μg of AN1792 in 200 μl PBS was emulsified 1:1 (vol:vol) with Complete Freund's Adjuvant (CFA) in a final volume of 400 μl for the first immunization. For subsequent immunizations, the antigen was similarly emulsified with Incomplete Freund's Adjuvant (IFA). For the formulations containing the adjuvants alum, MPL or QS-21, 100 μg per dose of AN1792 or AN1528 was combined with alum (1 mg per dose) or MPL (50 μg per dose) or QS-21 (25 μg per dose) in a final volume of 200 μl PBS and delivered by subcutaneous inoculation on the back between the shoulder blades. For the group receiving FA, 100 μg of AN1792 was emulsified 1:1 (vol:vol) with Complete Freund's adjuvant (CFA) in a final volume of 400 μl and delivered intraperitoneally for the first immunization, followed by a boost of the same amount of immunogen in Incomplete Freund's adjuvant (IFA) for the subsequent five doses. For the group receiving AN1792 without adjuvant, 10 μg AN1792 was combined with 5 μg thimerosal in a final volume of 50 μl PBS and delivered subcutaneously. The ninth, control group received only 200 μl PBS delivered subcutaneously. Immunizations were given on a biweekly schedule for the first three doses, then on a monthly schedule thereafter on days 0, 16, 28, 56, 85 and 112. Animals were bled six to seven days following each immunization starting after the second dose for the measurement of antibody titers. Animals were euthanized approximately one week after the final dose. Outcomes were measured by ELISA assay of Aβ and APP levels in brain and by immunohistochemical evaluation of the presence of amyloid plaques in brain sections. In addition, Aβ-specific antibody titers, and Aβ-dependent proliferative and cytokine responses were determined.

Table 9 shows that the highest antibody titers to Aβ1-42 were elicited with FA and AN1792, titers which peaked following the fourth immunization (peak GMT: 75,386) and then declined by 59% after the final, sixth immunization. The peak mean titer elicited by MPL with AN1792 was 62% lower than that generated with FA (peak GMT: 28,867) and was also reached early in the immunization scheme, after 3 doses, followed by a decline to 28% of the peak value after the sixth immunization. The peak mean titer generated with QS-21 combined with AN1792 (GMT: 1,511) was about 5-fold lower than obtained with MPL. In addition, the kinetics of the response were slower, since an additional immunization was required to reach the peak response. Titers generated by alum-bound AN1792 were marginally greater than those obtained with QS-21 and the response kinetics were more rapid. For AN1792 delivered in PBS with thimerosal the frequency and size of titers were barely greater than that for PBS alone. The peak titers generated with MPL and AN1528 (peak GMT 3099) were about 9-fold lower than those with AN1792. Alum-bound AN1528 was very poorly immunogenic with low titers generated in only some of the animals. No antibody responses were observed in the control animals immunized with PBS alone.

Table 9

Geometric Mean Antibody Titers[a]					
	Week of Bleed				
Treatment	3.3	5.0	9.0	13.0	17.0
Alum/ AN1792	102 (12/21)[b]	1,081 (17/20)	2,366 (21/21)	1,083 (19/21)	572 (18/21)
MPL/ AN1792	6241 (21/21)	28,867 (21/21)	1,1242 (21/21)	5,665 (20/20)	8,204 (20/20)
QS-21/ AN1792	30 (1/20)	227 (10/19)	327 (10/19)	1,511 (17/18)	1,188 (14/18)
CFA/ AN1792	10,076 (15/15)	61,279 (15/15)	75,386 (15/15)	41,628 (15/15)	30,574 (15/15)
Alum/ AN1528	25 (0/21)	33 (1/21)	39 (3/20)	37 (1/20)	31 (2/20)
MPL/ AN1528	184 (15/21)	2,591 (20/21)	1,653 (21/21)	1,156 (20/20)	3,099 (20/20)
QS-21/ AN1528	29 (1/22)	221 (13/22)	51 (4/22)	820 (20/22)	2,994 (21/22)
PBS plus Thimerosal	25 (0/16)	33 (2/16)	39 (4/16)	37 (3/16)	47 (4/16)
PBS	25 (0/16)	25 (0/16)	25 (0/15)	25 (0/12)	25 (0/16)

Footnotes:

[a] Geometric mean antibody titers measured against Aβ1-42

[b] Number of responders per group

The results of AN1792 or AN1528 treatment with various adjuvants, or thimerosal on cortical amyloid burden in 12-month old mice determined by ELISA are shown in Fig. 15. In PBS control PDAPP mice, the median level of total Aβ in the cortex at 12 months was 1,817 ng/g. Notably reduced levels of Aβ were observed in mice treated with AN1792 plus CFA/IFA, AN1792 plus alum, AN1792 plus MPL and QS-21 plus AN1792. The reduction reached statistical significance ($p<0.05$) only for AN1792 plus CFA/IFA. However, as shown in Examples I and III, the effects of immunization in reducing Aβ levels become substantially greater in 15 month and 18 month old mice. Thus, it is expected that at least the AN1792 plus alum, AN1792 plus MPL and AN1792 plus QS-21 compositions will achieve statistical significance in treatment of older mice. By contrast, the AN1792 plus the preservative thimerosal showed a median level of Aβ about the same as that in the PBS treated mice. Similar results were obtained when cortical levels of Aβ42 were compared. The median level of Aβ42 in PBS controls was 1624 ng/g. Notably reduced median levels of 403, 1149, 620 and 714 were observed in the mice treated with AN1792 plus CFA/IFA, AN1792 plus alum, AN1792 plus MPL and AN1792 plus QS-21 respectively, with the reduction achieving statistical significance ($p=0.05$) for the AN1792 CFA/IFA treatment group. The median level in the AN1792 thimerosal treated mice was 1619 ng/g Aβ42.

A further therapeutic adjuvant/immunogen efficacy study was performed in 9 - 10.5 month old male and female heterozygous PDAPP transgenic mice. The duration of the study was 25 weeks with 29–40 animals per treatment group; therefore the animals were 15 – 16.5 months old at termination.. The treatment groups are identified in Table 10 below.

	Adjuvant	Immunogen	Dilution Buffer	Administration
Group 1:	MPL-SE	AN1792-GCS (75 μg)	PBS	SC (250 μl)
Group 2:	ISA 51	AN1792-GCS (75 μg)	PBS	IP (400 μl)
Group 3:	QS21	AN1792-GCS (75 μg)	PBS	SC (250 μl)
Group 4:	QS21 abbrev.	AN1792-GCS (75 μg)	PBS	SC (250 μl)
Group 5:	PBS	------	------	SC (250 μl)

Table 10 abbreviations: MAP – multi-antigenic peptide; TT – tetanus toxoid t-cell epitope (830-844); SQ – subcutaneous; IP – intraperitoneally; PBS – phosphate, buffered saline; ISA-51 is a commercially available adjuvant similar to IFA; GCS is a glycine/citrate/sucrose formulation, MPL-SE is MPL in a stabilized water and oil emulsion.

The immunization schedule was identical for all of the treatment groups except for Group 3, the QS21/AN1792 abbreviated schedule group. The mice were injected on weeks 0, 2, 4, 8, 12, 16, 20, 24, with bleeds on weeks 3, 5, 9, 13, 17, 21 and 25. Groups 1, 2, received eight injections and Group 3 received four injections during the 25-week period of the study. Group 4, the QS21/AN1792 abbreviated schedule, received injections on weeks 0, 2, 4, and 8 only. This group was not injected for the remainder of the study, although they were bled on the same bleed schedule as the rest of the study to follow titer decay. Groups 3 and 5, QS21/AN1792 and PBS respectively, served as the positive and negative controls for this study.

The titers were determined by the anti-Aß antibody titer assay.

Group 1, the MPL-SE/AN1792 group, raised a peak geometric mean titer (GMT) of 17,100 at 9 weeks falling to a GMT of 10,000 at 25 weeks. Initially, the MPL-SE titers rose at a somewhat higher rate than the QS21/AN1792 control group (Group 4).

Group 2, the ISA 51/AN1792 group, produced high titers throughout the study reaching a GMT of over 100,000 for the last 9 weeks of the study.

Group 3, the QS21/AN1792 control group, reached its peak titer at 17 weeks with a GMT of 16,000. The titer then fell over the next 8 weeks to finish with a GMT of 8,700. One animal in this group failed to raise a titer over the entire course of the experiment.

Group 4, the QS21/AN1792 abbreviated injection schedule group, reached a peak titer of 7,300 at 13 weeks, five weeks after its final injection. The titer then fell to a GMT of 2,100 at the final bleed (25 weeks). As in the control group, one animal failed to raise a detectable titer, while another animal lost all titer by the end of the decay period.

Group 5, the PBS alone group, had no titers.

To evaluate the cortical Aß levels, total Aß and Aß1-42 were measured by ELISA. Briefly, one brain hemisphere was dissected for cortical, hippocampal, and cerebellar tissue followed by homogenization in 5M guanidine buffer and assayed for brain Aß. The cortical total Aß and Aß42 results are similar. A Mann-Whitney statistical analysis was performed to determine significance between the groups with a p value of 0.05 indicating a significant change in Aß.

All treatment groups significantly lowered total Aß levels as compared to the PBS control group (see Table 11). The MPL-SE/AN1792 group, showed the greatest change in Aß, and it is significantly better than the other treatment groups. The QS21/AN1792 abbreviated group, was similar in its overall change of Aß to the QS21 control group that received all eight injections. The Aß levels in the ISA 51/AN1792 group, were similarly lowered compared to the CFA/IFA:MAP(Aß1-7) group.

Table 11 Cortical Aß levels

	PBS	MPL-SE	ISA	QS-21	QS-21 (4)
MEDIAN (ng/g tissue)	7,335	1,236	3,026	2,389	2,996
RANGE (ng/g tissue)	550 – 18,358	70 – 3,977	23 – 9,777	210 – 11,167	24 – 16,834
p value	----	< 0.0001	< 0.0001	< 0.0001	< 0.0001
N	38	29	36	34	40

In conclusion, MPL-SE, ISA-51 and QS21 adjuvants combined with AN1792 are effective in inducing a sufficient immune response significantly to retard Aß deposition in the cortex.

X. Toxicity Analysis

Tissues were collected for histopathologic examination at the termination of studies described in Examples 2, 3 and 7. In addition, hematology and clinical chemistry were performed on terminal blood samples from Examples 3 and 7. Most of the major organs were evaluated, including brain, pulmonary, lymphoid, gastrointestinal, liver, kidney, adrenal and gonads. Although sporadic lesions were observed in the study animals, there were no obvious differences, either in tissues affected or lesion severity, between AN1792 treated and untreated animals. There were no unique histopathological lesions noted in AN-1528-immunized animals compared to PBS-treated or untreated animals. There were also no differences in the clinical chemistry profile between adjuvant groups and the PBS treated animals in Example 7. Although there were significant increases in several of the hematology parameters between animals treated with AN1792 and Freund's adjuvant in Example 7 relative to PBS treated animals, these type of effects are expected from Freund's adjuvant treatment and the accompanying peritonitis and do not indicate any adverse effects from AN1792 treatment. Although not part of the toxicological evaluation, PDAPP mouse brain pathology was extensively examined as part of the efficacy endpoints. No sign of treatment related adverse effect on brain morphology was noted in any of the studies. These results indicate that AN1792 treatment is well tolerated and at least substantially free of side effects.

XI. Therapeutic Treatment with Anti-Aβ antibodies

This examples tests the capacity of various monoclonal and polyclonal antibodies to Aβ to inhibit accumulation of Aβ in the brain of heterozygotic transgenic mice.

1. Study Design

Sixty male and female, heterozygous PDAPP transgenic mice, 8.5 to 10.5 months of age were obtained from Charles River Laboratory. The mice were sorted into six groups to be treated with various antibodies directed to Aβ. Animals were distributed to match the gender, age, parentage and source of the animals within the groups as closely as possible. As shown in Table 10, the antibodies included four murine Aβ-specific monoclonal antibodies, 2H3 (directed to Aβ residues 1-12), 10D5 (directed to Aβ residues 1-16), 266 (directed to Aβ residues 13-28 and binds to monomeric but not to aggregated AN1792), 21F12 (directed to Aβ residues 33-42). A fifth group was treated with an Aβ-specific polyclonal antibody fraction (raised by immunization with aggregated AN1792). The negative control group received the diluent, PBS, alone without antibody.

The monoclonal antibodies were injected at a dose of about 10 mg/kg (assuming that the mice weighed 50 g). Injections were administered intraperitoneally every seven days on average to maintain anti-Aβ titers above 1000. Although lower titers were measured for mAb 266 since it does not bind well to the aggregated AN1792 used as the capture antigen in the assay, the same dosing schedule was maintained for this group. The group receiving monoclonal antibody 2H3 was discontinued within the first three weeks since the antibody was cleared too rapidly in vivo. Animals were bled prior to each dosing for the measurement of antibody titers. Treatment was continued over a six-month period for a total of 196 days. Animals were euthanized one week after the final dose.

Table 12

EXPERIMENTAL DESIGN

Treatment Group	N[a]	Treatment Antibody	Antibody Specificity	Antibody Isotype
1	9	none (PBS alone)	NA[b]	NA
2	10	Polyclonal	Aβ1-42	mixed
3	0	mAb[c] 2H3	Aβ1-12	IgG1
4	8	mAb 10D5	Aβ1-16	IgG1
5	6	mAb 266	Aβ13-28	IgG1
6	8	mAb 21F12	Aβ33-42	IgG2a

Footnotes

[a] Number of mice in group at termination of the experiment. All groups started with 10 animals per group.

[b] NA: not applicable

[c] mAb: monoclonal antibody

2. Materials and Methods

a. Preparation of the Antibodies

The anti-Aβ polyclonal antibody was prepared from blood collected from two groups of animals. The first group consisted of 100 female Swiss Webster mice, 6 to 8 weeks of age. They were immunized on days 0, 15, and 29 with 100 μg of AN1792 combined with CFA/IFA. A fourth injection was given on day 36 with one-half the dose of AN1792. Animals were exsanguinated upon sacrifice at day 42, serum was prepared and the sera were pooled to create a total of 64 ml. The second group consisted of 24 female mice isogenic with the PDAPP mice but nontransgenic for the human APP gene, 6 to 9 weeks of age. They were immunized on days 0, 14, 28 and 56 with 100 μg of AN1792 combined with CFA/IFA. These animals were also exsanguinated upon sacrifice at day 63, serum was prepared and pooled for a total of 14 ml. The two lots of sera were pooled. The antibody fraction was purified using two sequential rounds of precipitation with 50% saturated ammonium sulfate. The final precipitate was dialyzed against PBS and tested for endotoxin. The level of endotoxin was less than 1 EU/mg.

The anti-Aβ monoclonal antibodies were prepared from ascites fluid. The fluid was first delipidated by the addition of concentrated sodium dextran sulfate to ice-cold ascites fluid by stirring on ice to a reach a final concentration of 0.238%. Concentrated $CaCl_2$ was then added with stirring to reach a final concentration of 64mM. This solution was centrifuged at 10,000 x g and the pellet was discarded. The supernatant was stirred on ice with an equal volume of saturated ammonium sulfate added dropwise. The solution was centrifuged again at 10,000 x g and the supernatant was discarded. The pellet was resuspended and dialyzed against 20 mM Tris-HCl , 0.4 M NaCl, pH 7.5. This fraction was applied to a Pharmacia FPLC Sepharose Q Column and eluted with a reverse gradient from 0.4 M to 0.275 M NaCl in 20 mM Tris-HCl, pH 7.5.

The antibody peak was identified by absorbance at 280 nm and appropriate fractions were pooled. The purified antibody preparation was characterized by measuring the protein concentration using the BCA method and the purity using SDS-PAGE. The pool was also tested for endotoxin. The level of endotoxin was less than 1 EU/mg. titers, titers less than 100 were arbitrarily assigned a titer value of 25.

3. Aβ and APP Levels in the Brain:

Following about six months of treatment with the various anti-Aβ antibody preparations, brains were removed from the animals following saline perfusion. One hemisphere was prepared for immunohistochemical analysis and the second was used for the quantitation of Aβ and APP levels. To measure the concentrations of various forms of beta amyloid peptide and amyloid precursor protein (APP), the hemisphere was dissected and homogenates of the hippocampal, cortical, and cerebellar regions were prepared in 5M guanidine. These were serially diluted and the level of amyloid peptide or APP was quantitated by comparison to a series of dilutions of standards of Aβ peptide or APP of known concentrations in an ELISA format.

The levels of total Aβ and of Aβ1-42 measured by ELISA in homogenates of the cortex, and the hippocampus and the level of total Aβ in the cerebellum are shown in Tables 11, 12, and 13, respectively. The median concentration of total Aβ for the control group, inoculated with PBS, was 3.6-fold higher in the hippocampus than in the cortex (median of 63,389 ng/g hippocampal tissue compared to 17,818 ng/g for the cortex). The median level in the cerebellum of the control group (30.6 ng/g tissue) was more than 2,000-fold lower than in the hippocampus. These levels are similar to those that we have previously reported for heterozygous PDAPP transgenic mice of this age (Johnson-Wood et al., 1997).

For the cortex, one treatment group had a median Aβ level, measured as Aβ1-42, which differed significantly from that of the control group ($p < 0.05$), those animals receiving the polyclonal anti-Aβ antibody as shown in Table 13. The median level of Aβ1-42 was reduced by 65%, compared to the control for this treatment group. The median levels of Aβ1-42 were also significantly reduced by 55% compared to the control in one additional treatment group, those animals dosed with the mAb 10D5 ($p = 0.0433$).

Table 13

CORTEX

Treatment Group	N[a]	Medians						Means	
		Total Aβ			Aβ42			Total Aβ	Aβ42
		LISA value[b]	P value[c]	% Change	ELISA value	P value	% Change	ELISA value	ELISA value
PBS	9	17818	NA[d]	NA	13802	NA	NA	16150+/-7456[e]	12621+/-5738
Polyclonal anti-Aβ42	10	6160	0.0055	-65	4892	0.0071	-65	5912+/-4492	4454+/-3347
mAb 10D5	8	7915	0.1019	-56	6214	0.0433	-55	9695+/-6929	6943+/-3351
mAb 266	6	9144	0.1255	-49	8481	0.1255	-39	9204+/-9293	7489+/-6921
mAb 21F12	8	15158	0.2898	-15	13578	0.7003	-2	12481+/-7082	11005+/-6324

Footnotes:

a. Number of animals per group at the end of the experiment

b. ng/g tissue

c. Mann Whitney analysis

d. NA: not applicable

e. Standard Deviation

In the hippocampus, the median percent reduction of total Aβ associated with treatment with polyclonal anti-Aβ antibody (50%, $p = 0.0055$) was not as great as that observed in the cortex (65%) (Table 14). However, the absolute magnitude of the reduction was almost 3-fold greater in the hippocampus than in the cortex, a net reduction of 31,683 ng/g tissue in the hippocampus versus 11,658 ng/g tissue in the cortex. When measured as the level of the more amyloidogenic form of Aβ, Aβ1-42, rather than as total Aβ, the reduction achieved with the polyclonal antibody was significant ($p = 0.0025$). The median levels in groups treated with the mAbs 10D5 and 266 were reduced by 33% and 21%, respectively.

Table 14

HIPPOCAMPUS

Treatment Group	N[a]	Medians						Means	
		Total Aβ			Aβ42			Total Aβ	Aβ42
		ELISA value[b]	P value[c]	% Change	ELISA value	P value	% Change	ELISA value	ELISA value
PBS	9	63389	NA[d]	NA	54429	NA	NA	58351+/-13308[e]	52801+/-14701
Polyclonal anti-Aβ42	10	31706	0.0055	-50	27127	0.0025	-50	30058+/-22454	24853+/-18262
mAb 10D5	8	46779	0.0675	-26	36290	0.0543	-33	44581+/-18632	36465+/-17146
mAb 266	6	48689	0.0990	-23	43034	0.0990	-21	36419+/-27304	32919+/-25372
mAb 21F12	8	51563	0.7728	-19	47961	0.8099	-12	57327+/-28927	50305+/-23927

Footnotes:

a. Number of animals per group at the end of the experiment

b. ng/g tissue

c. Mann Whitney analysis

d. NA: not applicable

e. Standard Deviation

Total Aβ was also measured in the cerebellum (Table 15). Those groups dosed with the polyclonal anti-Aβ and the 266 antibody showed significant reductions of the levels of total Aβ (43% and 46%, $p = 0.0033$ and $p = 0.0184$, respectively) and that group treated with 10D5 had a near significant reduction (29%, $p = 0.0675$).

Table 15

CEREBELLUM					
Treatment Group	N[a]	Medians			Means
		Total Aβ			Total Aβ
		ELISA value[b]	P value[c]	% Change	ELISA value
PBS	9	30.64	NA[d]	NA	40.00+/-31.89[e]
Polyclonal anti-Aβ42	10	17.61	0.0033	-43	18.15+/-4.36
mAb 10D5	8	21.68	0.0675	-29	27.29+/-19.43
mAb 266	6	16.59	0.0184	-46	19.59+/-6.59
mAb 21F12	8	29.80	>0.9999	-3	32.88+/-9.90

Footnotes:

a. Number of animals per group at the end of the experiment

b. ng/g tissue

c. Mann Whitney analysis

d. NA: not applicable

e. Standard Deviation

APP concentration was also determined by ELISA in the cortex and cerebellum from antibody-treated and control, PBS-treated mice. Two different APP assays were utilized. The first, designated APP-α/FL, recognizes both APP-alpha (α, the secreted form of APP which has been cleaved within the Aβ sequence), and full-length forms (FL) of APP, while the second recognizes only APP-α. In contrast to the treatment-associated diminution of Aβ in a subset of treatment groups, the levels of APP were virtually unchanged in all of the treated compared to the control animals. These results indicate that the immunizations with Aβ antibodies deplete Aβ without depleting APP.

In summary, Aβ levels were significantly reduced in the cortex, hippocampus and cerebellum in animals treated with the polyclonal antibody raised against AN1792. To a lesser extent monoclonal antibodies to the amino terminal region of Aβ1-42, specifically amino acids 1-16 and 13-28 also showed significant treatment effects.

4. Histochemical Analyses:

The morphology of Aß-immunoreactive plaques in subsets of brains from mice in the PBS, polyclonal Aß42, 21F12, 266 and 10D5 treatment groups was qualitatively compared to that of previous studies in which standard immunization procedures with Aß42 were followed.

The largest alteration in both the extent and appearance of amyloid plaques occurred in the animals immunized with the polyclonal Aß42 antibody. The reduction of amyloid load, eroded plaque morphology and cell-associated Aß immunoreactivity closely resembled effects produced by the standard immunization procedure. These observations support the ELISA results in which significant reductions in both total Aß and Aß42 were achieved by administration of the polyclonal Aß42 antibody.

In similar qualitative evaluations, amyloid plaques in the 10D5 group were also reduced in number and appearance, with some evidence of cell-associated Aß immunoreactivity. Relative to control-treated animals, the polyclonal Ig fraction against Aß and one of the monoclonal antibodies (10D5) reduced plaque burden by 93% and 81%, respectively ($p<0.005$). 21F12 appeared to have a relatively modest effect on plaque burden. Micrographs of brain after treatment with pabAß$_{1-42}$ show diffuse deposits and absence of many of the larger compacted plaques in the pabAß$_{1-42}$ treated group relative to control treated animals.

5. Measurement of Antibody Titers:

A subset of three randomly chosen mice from each group were bled just prior to each intraperitoneal inoculation, for a total of 30 bleeds. Antibody titers were measured as Aβ1-42-binding antibody using a sandwich ELISA with plastic multi-well plates coated with Aβ1-42 as described in detail in the General Materials and Methods. Mean titers for each bleed are shown in Figures 16-18 for the polyclonal antibody and the monoclonals 10D5 and 21F12, respectively. Titers averaged about 1000 over this time period for the polyclonal antibody preparation and were slightly above this level for the 10D5- and 21F12-treated animals.

6. Lymphoproliferative Responses:

Aβ-dependent lymphoproliferation was measured using spleen cells harvested eight days following the final antibody infusion. Freshly harvested cells, 10^5 per well, were cultured for 5 days in the presence of Aβ1-40 at a concentration of 5 μM for stimulation. As a positive control, additional cells were cultured with the T cell mitogen, PHA, and, as a negative control, cells were cultured without added peptide.

Splenocytes from aged PDAPP mice passively immunized with various anti-Aβ antibodies were stimulated *in vitro* with AN1792 and proliferative and cytokine responses were measured. The purpose of these assays was to determine if passive immunization facilitated antigen presentation, and thus priming of T cell responses specific for AN1792. No AN1792-specific proliferative or cytokine responses were observed in mice passively immunized with the anti-Aβ antibodies.

XII: FURTHER STUDY OF PASSIVE IMMUNIZATION

In a second study, treatment with 10D5 was repeated and two additional anti-Aß antibodies were tested, monoclonals 3D6 (Aß$_{1-5}$) and 16C11 (Aß$_{33-42}$). Control groups received either PBS or an irrelevant isotype-matched antibody (TM2a). The mice were older (11.5-12 month old heterozygotes) than in the previous study, otherwise the experimental design was the same. Once again, after six months of treatment, 10D5 reduced plaque burden by greater than 80% relative to either the PBS or isotype-matched antibody controls ($p=0.003$). One of the other antibodies against Aß, 3D6, was equally effective, producing an 86% reduction ($p=0.003$). In contrast, the third antibody against the peptide, 16C11, failed to have any effect on plaque burden. Similar findings were obtained with Aß$_{42}$ ELISA measurements. These results demonstrate that an antibody response against Aß peptide, in the absence of T cell immunity, is sufficient to decrease amyloid deposition in PDAPP mice, but that not all anti-Aß antibodies are efficacious. Antibodies directed to epitopes comprising amino acids 1-5 or 3-7 of Aβ are particularly efficacious.

In summary, we have shown that passively administered antibodies against Aß reduced the extent of plaque deposition in a mouse model of Alzheimer's disease. When held at modest serum concentrations (25–70 µg/ml), the antibodies gained access to the CNS at levels sufficient to decorate ß-amyloid plaques. Antibody entry into the CNS was not due to abnormal leakage of the blood-brain barrier since there was no increase in vascular permeability as measured by Evans Blue in PDAPP mice. In addition, the concentration of antibody in the brain parenchyma of aged PDAPP mice was the same as in non-transgenic mice, representing 0.1% of the antibody concentration in serum (regardless of isotype).

XIII: MONITORING OF ANTIBODY BINDING

To determine whether antibodies against Aß could be acting directly within the CNS, brains taken from saline-perfused mice at the end of the Example XII, were examined for the presence of the peripherally-administered antibodies. Unfixed cryostat brain sections were exposed to a fluorescent reagent against mouse immunoglobulin (goat anti-mouse IgG-Cy3). Plaques within brains of the 10D5 and 3D6 groups were strongly decorated with antibody, while there was no staining in the 16C11 group. To reveal the full extent of plaque deposition, serial sections of each brain were first immunoreacted with an anti-Aß antibody, and then with the secondary reagent. 10D5 and 3D6, following peripheral administration, gained access to most plaques within the CNS. The plaque burden was greatly reduced in these treatment groups compared to the 16C11 group. These data indicate that peripherally administered antibodies can enter the CNS where they can directly trigger amyloid clearance. It is likely that 16C11 also had access to the plaques but was unable to bind.

XIV: EX VIVO SCREENING ASSAY FOR ACTIVITY OF AN ANTIBODY AGAINST AMYLOID DEPOSITS

To examine the effect of antibodies on plaque clearance, we established an *ex vivo* assay in which primary microglial cells were cultured with unfixed cryostat sections of either PDAPP mouse or human AD brains. Microglial cells were obtained from the cerebral cortices of neonate DBA/2N mice (1-3 days). The cortices were mechanically dissociated in HBSS^{--} (Hanks' Balanced Salt Solution, Sigma) with 50 µg/ml DNase I (Sigma). The dissociated cells were filtered with a 100 µm cell strainer (Falcon), and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in growth medium (high glucose DMEM, 10%FBS, 25ng/ml rmGM-CSF), and the cells were plated at a density of 2 brains per T-75 plastic culture flask. After 7-9 days, the flasks were rotated on an orbital shaker at 200 rpm for 2h at 37°C. The cell suspension was centrifuged at 1000rpm and resuspended in the assay medium.

10-µm cryostat sections of PDAPP mouse or human AD brains (post-mortem interval < 3hr) were thaw mounted onto poly-lysine coated round glass coverslips and placed in wells of 24-well tissue culture plates. The coverslips were washed twice with assay medium consisting of H-SFM (Hybridoma-serum free medium, Gibco BRL) with 1% FBS, glutamine, penicillin/streptomycin, and 5ng/ml rmGM-CSF (R&D). Control or anti-Aß antibodies were added at a 2x concentration (5 µg/ml final) for 1 hour. The microglial cells were then seeded at a density of 0.8x 10^6 cells/ml assay medium. The cultures were maintained in a humidified incubator (37°C, 5%CO_2) for 24hr or more. At the end of the incubation, the cultures were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X100. The sections were stained with biotinylated 3D6 followed by a streptavidin / Cy3 conjugate (Jackson ImmunoResearch). The exogenous microglial cells were visualized by a nuclear stain (DAPI). The cultures were observed with an inverted fluorescent microscope (Nikon, TE300) and photomicrographs were taken with a SPOT digital camera using SPOT software (Diagnostic instruments). For Western blot analysis, the cultures were extracted in 8M urea, diluted 1:1 in reducing tricine sample buffer and loaded onto a 16% tricine gel (Novex). After transfer onto immobilon, blots were exposed to 5 µg/ml of the pabAß42 followed by an HRP-conjugated anti-mouse antibody, and developed with ECL (Amersham)

When the assay was performed with PDAPP brain sections in the presence of 16C11 (one of the antibodies against Aß that was not efficacious *in vivo*), ß-amyloid plaques remained intact and no phagocytosis was observed. In contrast, when adjacent sections were cultured in the presence of 10D5, the amyloid deposits were largely gone and the microglial cells showed numerous phagocytic vesicles containing Aß. Identical results were obtained with AD brain sections; 10D5 induced phagocytosis of AD plaques, while 16C11 was ineffective. In addition, the assay provided comparable results when performed with either mouse or human microglial cells, and with mouse, rabbit, or primate antibodies against Aß.

Table 16 shows whether binding and/or phagocytosis was obtained for several different antibody binding specificities. It can be seen that antibodies binding to epitopes within aa 1-7 both bind and clear amyloid deposits, whereas antibodies binding to epitopes within amino acids 4-10 bind without clearing amyloid deposits. Antibodies binding to epitopes C-terminal to residue 10 neither bind nor clear amyloid deposits.

Table 16: Analysis of Epitope Specificity

	Antibody		Staining	Phagocytosis
	epitope	isotype		
N-Term				
mab				
3D6	1-5	IgG2b	+	+
10D5	3-6	IgG1	+	+
22C8	3-7	IgG2a	+	+
6E10	5-10	IgG1	+	-
14A8	4-10	rat IgG1	+	-
13-28				
18G11	10-18	rat IgG1	-	-
266	16-24	IgG1	-	-
22D12	18-21	IgG2b	-	-
C-Term				
2G3	-40	IgG1	-	-
16C11	-40/-42	IgG1	-	-
21F12	-42	IgG2a	-	-
Immune serum				
rabbit (CFA)	1-6		+	+
mouse (CFA)	3-7		+	+
mouse (QS-21)	3-7		+	+
monkey (QS-21)	1-5		+	+
mouse (MAP1-7)			+	+

Table 17 shows results obtained with several antibodies against Aß, comparing their abilities to induce phagocytosis in the *ex vivo* assay and to reduce *in vivo* plaque burden in passive transfer studies. Although 16C11 and 21F12 bound to aggregated synthetic Aß peptide with high avidity, these antibodies were unable to react with ß-amyloid plaques in unfixed brain sections, could not trigger phagocytosis in the *ex vivo* assay, and were not efficacious *in vivo*. 10D5, 3D6, and the polyclonal antibody against Aß were active by all three measures. The 22C8 antibody binds more strongly to an analog form of natural

Aß in which aspartic acid at positions 1 and 7 is replaced with iso-aspartic acid. These results show that efficacy *in vivo* is due to direct antibody mediated clearance of the plaques within the CNS, and that the *ex vivo* assay is predictive of *in vivo* efficacy.

The same assay has been used to test clearing of an antibody against a fragment of synuclein referred to as NAC. Synuclein has been shown to be an amyloid plaque-associated protein. An antibody to NAC was contacted with a brain tissue sample containing amyloid plaques, an microglial cells, as before. Rabbit serum was used as a control. Subsequent monitoring showed a marked reduction in the number and size of plaques indicative of clearing activity of the antibody.

Table 17 The *ex vivo* assay as predictor of *in vivo* efficacy.

Antibody	**Isotype**	**Avidity for aggregated Aß (pM)**	**Binding to ß-amyloid plaques**	***Ex vivo* efficacy**	***In vivo* efficacy**
monoclonal					
3D6	IgG2b	470	+	+	+
10D5	IgG1	43	+	+	+
16C11	IgG1	90	-	-	-
21F12	IgG2a	500	-	-	-
TM2a	IgG1	-	-	-	-
polyclonal					
1-42	mix	600	+	+	+

Confocal microscopy was used to confirm that Aß was internalized during the course of the *ex vivo* assay. In the presence of control antibodies, the exogenous microglial cells remained in a confocal plane above the tissue, there were no phagocytic vesicles containing Aß, and the plaques remained intact within the section. In the presence of 10D5, nearly all plaque material was contained in vesicles within the exogenous microglial cells. To determine the fate of the internalized peptide, 10D5 treated cultures were extracted with 8M urea at various time-points, and examined by Western blot analysis. At the one hour time point, when no phagocytosis had yet occurred, reaction with a polyclonal antibody against Aß revealed a strong 4 kD band (corresponding to the Aß peptide). Aß immunoreactivity decreased at day 1 and was absent by day 3. Thus, antibody-mediated phagocytosis of Aß leads to its degradation.

To determine if phagocytosis in the *ex vivo* assay was Fc-mediated, F(ab')2 fragments of the anti-Aß antibody 3D6 were prepared. Although the F(ab')2 fragments retained their full ability to react with plaques, they were unable to trigger phagocytosis by microglial cells. In addition, phagocytosis with the whole antibody could be blocked by a reagent against murine Fc receptors (anti-CD16/32). These data indicate that *in vivo* clearance of Aß occurs through Fc-receptor mediated phagocytosis.

XV: PASSAGE OF ANTIBODIES THROUGH BLOOD BRAIN BARRIER

This example determines the concentration of antibody delivered to the brain following intravenous injection into a peripheral tissue of either normal or PDAPP mice. PDAPP or control normal mice were perfused with 0.9% NaCl. Brain regions (hippocampus or cortex) were dissected and rapidly frozen. Brain were homogenized in 0.1% triton + protease inhibitors. Immunoglobulin was detected in the extracts by ELISA. Fab'2 Goat Anti-mouse IgG were coated onto an RIA plate as capture reagent. The serum or the brain extracts were incubated for 1hr. The isotypes were detected with anti-mouse IgG1-HRP or IgG2a-HRP or IgG2b-HRP (Caltag). Antibodies, regardless of isotype, were present in the CNS at a concentration that is 1:1000 that found in the blood. For example, when the concentration of IgG1 was three times that of IgG2a in the blood, it was three times IgG2a in the brain as well, both being present at 0.1% of their respective levels in the blood. This result was observed in both transgenic and nontransgenic mice - so the PDAPP does not have a uniquely leak blood brain barrier.

XVI: THERAPEUTIC EFFICACY OF AN Aß PEPTIDE IN MAP CONFIGURATION

A therapeutic adjuvant/immunogen efficacy study was performed in 9 - 10.5 month old male and female heterozygous PDAPP transgenic mice to test the efficacy of a fusion protein comprising Aß1-7 in tetrameric MAP configuration as described above. The duration of the study was 25 weeks with 29 – 40 animals per treatment group; therefore the animals were 15 – 16.5 months old at termination. The methodology used in this study is the same as that in the therapeutic study of different adjuvants in Example VIII above. . The treatment groups are identified in Table 18 below.

Table 18

	Adjuvant	Immunogen	Dilution Buffer	Administration
Group 1:	CFA/IFA	MAP(Aß 1-7:TT) (100 μg)	PBS	IP (400 μl)
Group 2:	QS21	AN1792-GCS (75 μg)	PBS	SC (250 μl)
Group 3:	PBS	------	------	SC (250 μl)

Table abbreviations: MAP – multi-antigenic peptide; TT – tetanus toxoid t-cell epitope (830-844); SC – subcutaneous; IP – intraperitoneally; PBS – phosphate buffered saline; GCS is a glycine/citrate/sucrose formulation.

The immunization schedule was identical for all of the treatment groups. The mice were injected on weeks 0, 2, 4, 8, 12, 16, 20, 24, with bleeds on week 3, 5, 9, 13, 17, 21 and 25. Groups 1, 2, 3, 4, and 6 received eight injections Groups 2 and 3, QS21/AN1792 and PBS respectively, served as the positive and negative controls for this study.

The titers were determined by the anti-Aß antibody titer assay.

Group 1, CFA/IFA:MAP(Aß1-7:TT) group, had low titer levels. The peak GMT reached was only 1,200 at 13 weeks, falling to a GMT of 600 by week 25. There were 3 of the 30 mice that did not raise any titer and another 7 mice that did not exceed a titer of 400 by the end of the study.

Group 2, the QS21/AN1792 control group, reached its peak titer at 17 weeks with a GMT of 16,000. The titer then fell over the next 8 weeks to finish with a GMT of 8,700. One animal in this group failed to raise a titer over the entire course of the experiment.

Group 3, the PBS alone group, had no titers.

Both treatment groups showed a significant lowing in cortical Aß levels as compared to the PBS control group (see Table 19). The CFA/IFA:MAP(Aß1-7) group, significantly lowered Aß as compared to the PBS control group in spite of the relatively low titers of anti-Aß antibodies.

Table 19 Cortical Aß levels

	PBS	MAP	QS-21
MEDIAN (ng/g tissue)	7,335	3,692	2,389
RANGE (ng/g tissue)	550 – 18,358	240 – 10,782	210 – 11,167
p value	----	0.0003	< 0.0001
N	38	30	34

In conclusion, the Aß 1-7MAP immunogen is effective in inducing a sufficient immune response significantly to retard Aß deposition in the cortex.

XVII. EPITOPE MAPPING OF IMMUNOGENIC RESPONSE TO Aβ IN MONKEYS

This example analyzes the response of a primate to immunization with AN1792 (i.e., Aβ1-42). Eleven groups of monkeys (4/sex/group) were immunized with AN1792 (75 or 300 μg/dose) in combination with QS-21 adjuvant (50 or 100 μg/dose) or 5% sterile dextrose in water (D5W, control group). All animals received IM injections on one of three injection schedules as shown in Table 20 for a total of 4, 5 or 8 doses. Serum samples (from 4 monkeys/sex/group) collected on Day 175 of the study and CSF samples (from 3 monkeys/sex/group) collected on Day 176 of the study (at the 6 month necropsy) were evaluated for their ability to bind to Aβ1-40 peptide and APP.

Table 20: Group Assignments and Dose Levels

Group No.	Schedule[a]	# Monkeys (M/F)	AN1792 Dose (μg/dose)	QS-21 Dose (μg/dose)	Dose Route
1[b]	1	4/4	0	0	IM
2	1	4/4	Vehicle[c]	50	IM
3	1	4/4	Vehicle	100	IM
4	1	4/4	75	50	IM
5	1	4/4	300	50	IM
6	1	4/4	75	100	IM
7	1	4/4	300	100	IM
8	2	4/4	75	100	IM
9	2	4/4	300	100	IM
10	3	4/4	75	100	IM
11	3	4/4	300	100	IM

a. Schedule 1, Dose Days 1, 15, 29, 57, 85, 113, 141, 169; Schedule 2, Dose Days 1, 29, 57, 113, 169; Schedule 3, Dose Days 1, 43, 85, 169

b. D5W injection control group

c. Vehicle consists of the glycine/citrate/sucrose buffer which is the excipient for AN1792.

The exact array of linear peptides recognized by the antibodies in the serum samples from animals immunized with AN1792 was determined by an ELISA that measured the binding of these antibodies to overlapping peptides that covered the entire Aβ1-42 sequence. Biotinylated peptides with partial sequences of AN1792 were obtained from Chiron Technologies as 10 amino acid peptides with an overlap of 9 residues and a step of one residue per peptide (synthesis No. 5366, No. 5331 and No. 5814). The first 32 peptides (from the eight amino acid position upstream of the N-terminal of AN1792 down to the twenty-fourth amino acid of AN1792) are biotinylated on the C-terminal with a linker of GGK. The last 10 peptides (repeating the thirty-second peptide from the previous series) are biotinylated on the N-terminal with a linker consisting of EGEG). The lyophilized biotinylated peptides were dissolved at a concentration of 5 mM in DMSO. These peptide stocks were diluted to 5 μM in TTBS (0.05% Tween 20, 25 mM Tris HCl, 137 mM NaCl, 5.1 mM KCl, pH=7.5). 100 μl aliquots of this 5 μM solution were added in duplicate to streptavidin pre-coated 96-well plates (Pierce). Plates were incubated for one hour at room temperature, then washed four times with TTBS. Serum samples were diluted in specimen diluent without azide to normalize titers, and 100 μl was added per well. These plates were incubated one hour at room temperature and then washed four times with TTBS. HRP-conjugated goat anti-human antibody (Jackson ImmunoResearch) was diluted 1:10,000 in specimen diluent without azide and 100 µl was added per well. The plates were again incubated and washed. To develop the color reaction, TMB (Pierce), was added at 100 µl per well and incubated for 15 min prior to the addition of 30 µl of 2 N H_2SO_4 to stop the reaction. The optical density was measured at 450 nm on a Vmax or Spectramax colorimetric plate reader.

Immunization with AN1792 resulted in the production of antibodies in 100% of the animals in all of the dose groups by Day 175. Mean titers in the groups ranged from 14596 - 56084. There was a trend for titers to be higher within an immunization schedule in the presence of higher antigen and/or higher adjuvant concentration, but no statistically significant differences could be demonstrated due to the high variability in individual animal responses to the immunizations.

Sera which were positive for antibodies to AN1792 were also positive for antibodies to Aβ1-40. Mean titers in the groups ranged from 36867 - 165991, and as for anti-AN1792 titers, showed no statistically significant differences between groups at Day 175. Binding to AN1792 showed a highly positive correlation (Spearman r = 0.8671) with binding to Aβ1-40.

Of the 48 monkeys immunized on various schedules with AN1792, 33 yielded CSF samples of adequate volume and quality for analysis. Thirty-two (97%) of these monkeys had positive titers to AN1792. Titers ranged from 2-246, with a mean of 49.44 ± 21.34. CSF anti-AN1792 levels were 0.18 ± 0.11% of what was measured in the serum and demonstrated a highly positive correlation (Spearman r = 0.7840) with serum titers. No differences were seen across groups or between sexes in the percentage of antibody in the CSF. The level of antibody in the CSF is consistent with the passive transfer of peripherally generated antibody across the blood-brain-barrier into the central nervous system.

Testing of a subset of anti-AN1792 positive CSF samples demonstrated that, like the antibody in serum samples, antibody in the CSF cross-reacts with Aβ1-40. Titers to Aβ1-40 showed a high correlation (Spearman r = 0.9634) to their respective AN1792 titers. Testing of a subset of CSF samples with the highest titers to AN1792 showed no binding to APP, as for the serum antibodies.

When sera from Day 175 was tested against a series of overlapping 10-mer peptides, antibodies from all of the monkeys bound to the peptide whose sequence covered amino acids 1–10 of the AN1792 peptide (amino acids 653-672 of APP). In some animals, this was the only peptide to which binding could be measured (see Fig. 19).

In other animals, other reactivities could be measured, but in all cases the reactivity to the N-terminal peptide sequence was the predominant one. The additional reactivities fell into two groups. First and most common, was the binding to peptides centering around the N-terminal 1–10 AN1792 peptide (Figure 20). Binding of this type was directed to the peptides covering amino acids -1–8, -1–9, and 2–11 of the AN1792 peptide. These reactivities, combined with that to the 1–10 peptide, represent the overwhelming majority of reactivity in all animals. Epitope mapping of individual animals over time indicates that the antibody reactivity to the 1–10 peptide proceeds the spread to the adjacent peptides . This demonstrates a strong biasing of the immune response to the N-terminus of the AN1792 peptide with its free terminal aspartic acid residue. The second minor detectable activity in some animals was binding to peptides located C-terminally to the major area and centered around peptides covering amino acids 7–16, 11–20 and 16–25 of the AN1792 peptide . These reactivities were seen in only 10–30% of the monkeys.

Variability in response between different animals (e.g., whether amino acids 1-10 were the exclusive or predominant reactive epitope) did not correlate with antigen/adjuvant dose, dosing schedule, or antibody titer, and is probably a reflection of each individual animal's genetic make-up.

XVIII. PREVENTION AND TREATMENT OF HUMAN SUBJECTS

A single-dose phase I trial is performed to determine safety in humans. A therapeutic agent is administered in increasing dosages to different patients starting from about 0.01 the level of presumed efficacy, and increasing by a factor of three until a level of about 10 times the effective mouse dosage is reached.

A phase II trial is performed to determine therapeutic efficacy. Patients with early to mid Alzheimer's Disease defined using Alzheimer's disease and Related Disorders Association (ADRDA) criteria for probable AD are selected. Suitable patients score in the 12-26 range on the Mini-Mental State Exam (MMSE). Other selection criteria are that patients are likely to survive the duration of the study and lack complicating issues such as use of concomitant medications that may interfere. Baseline evaluations of patient function are made using classic psychometric measures, such as the MMSE, and the ADAS, which is a comprehensive scale for evaluating patients with Alzheimer's Disease status and function.

These psychometric scales provide a measure of progression of the Alzheimer's condition. Suitable qualitative life scales can also be used to monitor treatment. Disease progression can also be monitored by MRI. Blood profiles of patients can also be monitored including assays of immunogen-specific antibodies and T-cells responses.

Following baseline measures, patients begin receiving treatment. They are randomized and treated with either therapeutic agent or placebo in a blinded fashion. Patients are monitored at least every six months. Efficacy is determined by a significant reduction in progression of a treatment group relative to a placebo group.

A second phase II trial is performed to evaluate conversion of patients from non-Alzheimer's Disease early memory loss, sometimes referred to as age-associated memory impairment (AAMI) or mild cognitive impairment (MCI), to probable Alzheimer's disease as defined as by ADRDA criteria. Patients with high risk for conversion to Alzheimer's Disease are selected from a non-clinical population by screening reference populations for early signs of memory loss or other difficulties associated with pre-Alzheimer's symptomatology, a family history of Alzheimer's Disease, genetic risk factors, age, sex, and other features found to predict high-risk for Alzheimer's Disease. Baseline scores on suitable metrics including the MMSE and the ADAS together with other metrics designed to evaluate a more normal population are collected. These patient populations are divided into suitable groups with placebo comparison against dosing alternatives with the agent. These patient populations are followed at intervals of about six months, and the endpoint for each patient is whether or not he or she converts to probable Alzheimer's Disease as defined by ADRDA criteria at the end of the observation.

XIX. General Materials and Methods

1. Measurement of Antibody Titers

Mice were bled by making a small nick in the tail vein and collecting about 200 μl of blood into a microfuge tube. Guinea pigs were bled by first shaving the back hock area and then using an 18 gauge needle to nick the metatarsal vein and collecting the blood into microfuge tubes. Blood was allowed to clot for one hr at room temperature (RT), vortexed, then centrifuged at 14,000 x g for 10 min to separate the clot from the serum. Serum was then transferred to a clean microfuge tube and stored at 4°C until titered.

Antibody titers were measured by ELISA. 96-well microtiter plates (Costar EIA plates) were coated with 100 μl of a solution containing either 10 μg/ml either Aβ42 or

SAPP or other antigens as noted in each of the individual reports in Well Coating Buffer (0.1 M sodium phosphate, pH 8.5, 0.1% sodium azide) and held overnight at RT. The wells were aspirated and sera were added to the wells starting at a 1/100 dilution in Specimen Diluent (0.014 M sodium phosphate, pH 7.4, 0.15 M NaCl, 0.6% bovine serum albumin, 0.05% thimerosal). Seven serial dilutions of the samples were made directly in the plates in three-fold steps to reach a final dilution of 1/218,700. The dilutions were incubated in the coated-plate wells for one hr at RT. The plates were then washed four times with PBS containing 0.05% Tween 20. The second antibody, a goat anti-mouse Ig conjugated to horseradish peroxidase (obtained from Boehringer Mannheim), was added to the wells as 100 µl of a 1/3000 dilution in Specimen Diluent and incubated for one hr at RT. Plates were again washed four times in PBS, Tween 20. To develop the chromogen, 100 µl of Slow TMB (3,3',5,5'-tetramethyl benzidine obtained from Pierce Chemicals) was added to each well and incubated for 15 min at RT. The reaction was stopped by the addition of 25 µl of 2 M H_2SO_4. The color intensity was then read on a Molecular Devices Vmax at (450 nm - 650 nm).

Titers were defined as the reciprocal of the dilution of serum giving one half the maximum OD. Maximal OD was generally taken from an initial 1/100 dilution, except in cases with very high titers, in which case a higher initial dilution was necessary to establish the maximal OD. If the 50% point fell between two dilutions, a linear extrapolation was made to calculate the final titer. To calculate geometric mean antibody titers, titers less than 100 were arbitrarily assigned a titer value of 25.

2. Lymphocyte proliferation assay

Mice were anesthetized with isoflurane. Spleens were removed and rinsed twice with 5 ml PBS containing 10% heat-inactivated fetal bovine serum (PBS-FBS) and then homogenized in a 50° Centricon unit (Dako A/S, Denmark) in 1.5 ml PBS-FBS for 10 sec at 100 rpm in a Medimachine (Dako) followed by filtration through a 100 micron pore size nylon mesh. Splenocytes were washed once with 15 ml PBS-FBS, then pelleted by centrifugation at 200 x g for 5 min. Red blood cells were lysed by resuspending the pellet in 5 mL buffer containing 0.15 M NH4Cl, 1 M KHCO3, 0.1 M NaEDTA, pH 7.4 for five min at RT. Leukocytes were then washed as above. Freshly isolated spleen cells (10^5 cells per well) were cultured in triplicate sets in 96-well U-bottomed tissue culture-treated microtiter plates (Corning, Cambridge, MA) in RPMI 1640 medium (JRH Biosciences, Lenexa, KS) supplemented with 2.05 mM L glutamine, 1% Penicillin/Streptomycin, and 10% heat-inactivated FBS, for 96 hr at 37°C. Various Aβ peptides, Aβ1-16, Aβ1-40, Aβ1-42 or Aβ40-1 reverse sequence protein were also added at doses ranging from 5 to 0.18 micromolar in four steps. Cells in control wells were cultured with Concanavalin A (Con A) (Sigma, cat. # C-5275, at 1 microgram/ml) without added protein. Cells were pulsed for the final 24 hr with 3H-thymidine (1 μCi/well obtained from Amersham Corp., Arlington Heights IL). Cells were then harvested onto UniFilter plates and counted in a Top Count Microplate Scintillation Counter (Packard Instruments, Downers Grove, IL). Results are expressed as counts per minute (cpm) of radioactivity incorporated into insoluble macromolecules.

4. Brain Tissue Preparation

After euthanasia, the brains were removed and one hemisphere was prepared for immunohistochemical analysis, while three brain regions (hippocampus, cortex and cerebellum) were dissected from the other hemisphere and used to measure the concentration of various Aβ proteins and APP forms using specific ELISAs (Johnson-Wood et al., supra).

Tissues destined for ELISAs were homogenized in 10 volumes of ice-cold guanidine buffer (5.0 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0). The homogenates were mixed by gentle agitation using an Adams Nutator (Fisher) for three to four hr at RT, then stored at -20°C prior to quantitation of Aβ and APP. Previous experiments had shown that the analytes were stable under this storage condition, and that synthetic Aβ protein (Bachem) could be quantitatively recovered when spiked into homogenates of control brain tissue from mouse littermates (Johnson-Wood et al., supra).

5. Measurement of Aβ Levels

The brain homogenates were diluted 1:10 with ice cold Casein Diluent (0.25% casein, PBS, 0.05% sodium azide, 20 μg/ml aprotinin, 5 mM EDTA pH 8.0, 10 μg/ml leupeptin) and then centrifuged at 16,000 x g for 20 min at 4° C. The synthetic Aβ protein standards (1-42 amino acids) and the APP standards were prepared to include 0.5 M guanidine and 0.1% bovine serum albumin (BSA) in the final composition. The "total" Aβ sandwich ELISA utilizes monoclonal antibody monoclonal antibody 266, specific for amino acids 13-28 of Aβ (Seubert, et al.), as the capture antibody, and biotinylated monoclonal antibody 3D6, specific for amino acids 1-5 of Aβ (Johnson-Wood, et al), as the reporter antibody. The 3D6 monoclonal antibody does not recognize secreted APP or full-length APP, but detects only Aβ species with an amino-terminal aspartic acid. This assay has a lower limit of sensitivity of ~50 ng/ml (11nM) and shows no cross-reactivity to the endogenous murine Aβ protein at concentrations up to 1 ng/ml (Johnson-Wood et al., supra).

The Aβ1-42 specific sandwich ELISA employs mAβ 21F12, specific for amino acids 33-42 of Aβ (Johnson-Wood, et al.), as the capture antibody. Biotinylated mAβ 3D6 is also the reporter antibody in this assay which has a lower limit of sensitivity of about 125 μg/ml (28 μM, Johnson-Wood et al.). For the Aβ ELISAs, 100 μl of either mAβ 266 (at 10 μg/ml) or mAβ 21F12 at (5 μg/ml) was coated into the wells of 96-well immunoassay plates (Costar) by overnight incubation at RT. The solution was removed by aspiration and the wells were blocked by the addition of 200 μl of 0.25% human serum albumin in PBS buffer for at least 1 hr at RT. Blocking solution was removed and the plates were stored desiccated at 4°C until used. The plates were rehydrated with Wash Buffer [Tris-buffered saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5), plus 0.05% Tween 20] prior to use. The samples and standards were added in triplicate aliquots of 100 μl per well and then incubated overnight at 4° C. The plates were washed at least three times with Wash Buffer between each step of the assay. The biotinylated mAβ 3D6, diluted to 0.5 μg/ml in Casein Assay Buffer (0.25% casein, PBS, 0.05% Tween 20, pH 7.4), was added and incubated in the wells for 1 hr at RT. An avidin-horseradish peroxidase conjugate, (Avidin-HRP obtained from Vector, Burlingame, CA), diluted 1:4000 in Casein Assay Buffer, was added to the wells for 1 hr at RT. The colorimetric substrate, Slow TMB-ELISA (Pierce), was added and allowed to react for 15 minutes at RT, after which the enzymatic reaction was stopped by the addition of 25 μl 2 N H2SO4. The reaction product was quantified using a Molecular Devices Vmax measuring the difference in absorbance at 450 nm and 650 nm.

6. Measurement of APP Levels

Two different APP assays were utilized. The first, designated APP-α/FL, recognizes both APP-alpha (α) and full-length (FL) forms of APP. The second assay is specific for APP-α. The APP-α /FL assay recognizes secreted APP including the first 12 amino acids of Aβ. Since the reporter antibody (2H3) is not specific to the α-clip-site, occurring between amino acids 612-613 of APP695 (Esch et al., Science 248, 1122-1124 (1990)); this assay also recognizes full length APP (APP-FL). Preliminary experiments using immobilized APP antibodies to the cytoplasmic tail of APP-FL to deplete brain homogenates of APP-FL suggest that approximately 30-40% of the APP-α /FL APP is FL (data not shown). The capture antibody for both the APP-α/FL and APP-α assays is mAb 8E5, raised against amino acids 444 to 592 of the APP695 form (Games et al., supra). The reporter mAb for the APP-α/FL assay is mAb 2H3, specific for amino acids 597-608 of APP695 (Johnson-Wood et al., supra) and the reporter antibody for the APP-α assay is a biotinylated derivative of mAb 16H9, raised to amino acids 605 to 611 of APP. The lower limit of sensitivity of the APP-αFL assay is about 11 ng/ml (150 ρM) (Johnson-Wood et al.) and that of the APP-α specific assay is 22 ng/ml (0.3 nM). For both APP assays, mAb 8E5 was coated onto the wells of 96-well EIA plates as described above for mAb 266. Purified, recombinant secreted APP-α was used as the reference standard for the APP-α assay and the APP-α/FL assay (Esch et al., supra). The brain homogenate samples in 5 M guanidine were diluted 1:10 in ELISA Specimen Diluent (0.014 M phosphate buffer, pH 7.4, 0.6% bovine serum albumin, 0.05% thimerosal, 0.5 M NaCl, 0.1% NP40). They were then diluted 1:4 in Specimen Diluent containing 0.5 M guanidine. Diluted homogenates were then centrifuged at 16,000 x g for 15 seconds at RT. The APP standards and samples were added to the plate in duplicate aliquots and incubated for 1.5 hr at RT. The biotinylated reporter antibody 2H3 or 16H9 was incubated with samples for 1 hr at RT. Streptavidin-alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in specimen diluent, was incubated in the wells for 1 hr at RT. The fluorescent substrate 4-methyl-umbellipheryl-phosphate was added for a 30-min RT incubation and the plates were read on a Cytofluor tm 2350 fluorimeter (Millipore) at 365 nm excitation and 450 nm emission.

7. Immunohistochemistry

Brains were fixed for three days at 40C in 4% paraformaldehyde in PBS and then stored from one to seven days at 4°C in 1% paraformaldehyde, PBS until sectioned. Forty-micron-thick coronal sections were cut on a vibratome at RT and stored in cryoprotectant (30% glycerol, 30% ethylene glycol in phosphate buffer) at -20°C prior to immunohistochemical processing. For each brain, six sections at the level of the dorsal hippocampus, each separated by consecutive 240 μm intervals, were incubated overnight with one of the following antibodies: (1) a biotinylated anti-Aβ (mAb, 3D6, specific for human Aβ) diluted to a concentration of 2 μg/ml in PBS and 1% horse serum; or (2) a biotinylated mAb specific for human APP, 8E5, diluted to a concentration of 3 μg/ml in PBS and 1.0% horse serum; or (3) a mAb specific for glial fibrillary acidic protein (GFAP; Sigma Chemical Co.) diluted 1:500 with 0.25% Triton X-100 and 1% horse serum, in Tris-buffered saline, pH 7.4 (TBS); or (4) a mAb specific for CD11b, MAC-1 antigen, (Chemicon International) diluted 1:100 with 0.25% Triton X-100 and 1% rabbit serum in TBS; or (5) a mAb specific for MHC II antigen, (Pharmingen) diluted 1:100 with 0.25% Triton X-100 and 1% rabbit serum in TBS; or (6) a rat mAb specific for CD 43 (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS or (7) a rat mAb specific for CD 45RA (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (8) a rat monoclonal Aβ specific for CD 45RB (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (9) a rat monoclonal Aβ specific for CD 45 (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (10) a biotinylated polyclonal hamster Aβ specific for CD3e (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS or (11) a rat mAb specific for CD3 (Serotec) diluted 1:200 with 1% rabbit serum in PBS; or with (12) a solution of PBS lacking a primary antibody containing 1% normal horse serum.

Sections reacted with antibody solutions listed in 1,2 and 6-12 above were pretreated with 1.0% Triton X-100, 0.4% hydrogen peroxide in PBS for 20 min at RT to block endogenous peroxidase. They were next incubated overnight at 4°C with primary antibody. Sections reacted with 3D6 or 8E5 or CD3e mAbs were then reacted for one hr at RT with a horseradish peroxidase-avidin-biotin-complex with kit components "A" and "B" diluted 1:75 in PBS (Vector Elite Standard Kit, Vector Labs, Burlingame, CA.). Sections reacted with antibodies specific for CD 45RA, CD 45RB, CD 45, CD3 and the PBS solution devoid of primary antibody were incubated for 1 hour at RT with biotinylated anti-rat IgG (Vector) diluted 1:75 in PBS or biotinylated anti-mouse IgG (Vector) diluted 1:75 in PBS, respectively. Sections were then reacted for one hr at RT with a horseradish peroxidase-avidin-biotin-complex with kit components "A" and "B" diluted 1:75 in PBS (Vector Elite Standard Kit, Vector Labs, Burlingame, CA.).

Sections were developed in 0.01% hydrogen peroxide, 0.05% 3,3'-diaminobenzidine (DAB) at RT. Sections destined for incubation with the GFAP-, MAC-1- AND MHC II-specific antibodies were pretreated with 0.6% hydrogen peroxide at RT to block endogenous peroxidase then incubated overnight with the primary antibody at 4°C. Sections reacted with the GFAP antibody were incubated for 1 hr at RT with biotinylated anti-mouse IgG made in horse (Vector Laboratories; Vectastain Elite ABC Kit) diluted 1:200 with TBS. The sections were next reacted for one hr with an avidin-biotin-peroxidase complex (Vector Laboratories; Vectastain Elite ABC Kit) diluted 1:1000 with TBS. Sections incubated with the MAC-1-or MHC II-specific monoclonal antibody as the primary antibody were subsequently reacted for 1 hr at RT with biotinylated anti-rat IgG made in rabbit diluted 1:200 with TBS, followed by incubation for one hr with avidin-biotin-peroxidase complex diluted 1:1000 with TBS. Sections incubated with GFAP-, MAC-1- and MHC II-specific antibodies were then visualized by treatment at RT with 0.05% DAB, 0.01% hydrogen peroxide, 0.04% nickel chloride, TBS for 4 and 11 min, respectively.

Immunolabeled sections were mounted on glass slides (VWR, Superfrost slides), air dried overnight, dipped in Propar (Anatech) and overlaid with coverslips using Permount (Fisher) as the mounting medium.

To counterstain Aβ plaques, a subset of the GFAP-positive sections were mounted on Superfrost slides and incubated in aqueous 1% Thioflavin S (Sigma) for 7 min following immunohistochemical processing. Sections were then dehydrated and cleared in Propar, then overlaid with coverslips mounted with Permount.

8. Image Analysis

A Videometric 150 Image Analysis System (Oncor, Inc., Gaithersburg, MD) linked to a Nikon Microphot-FX microscope through a CCD video camera and a Sony Trinitron monitor was used for quantification of the immunoreactive slides. The image of the section was stored in a video buffer and a color-and saturation-based threshold was determined to select and calculate the total pixel area occupied by the immunolabeled structures. For each section, the hippocampus was manually outlined and the total pixel area occupied by the hippocampus was calculated. The percent amyloid burden was measured as: (the fraction of the hippocampal area containing Aβ deposits immunoreactive with mAb 3D6) x 100. Similarly, the percent neuritic burden was measured as: (the fraction of the hippocampal area containing dystrophic neurites reactive with monoclonal antibody 8E5) x100. The C-Imaging System (Compix, Inc., Cranberry Township, PA) operating the Simple 32 Software Application program was linked to a Nikon Microphot-FX microscope through an Optronics camera and used to quantitate the percentage of the retrospenial cortex occupied by GFAP-positive astrocytes and MAC-1-and MHC II-positive microglia. The image of the immunoreacted section was stored in a video buffer and a monochrome-based threshold was determined to select and calculate the total pixel area occupied by immunolabeled cells. For each section, the retrosplenial cortex (RSC) was manually outlined and the total pixel area occupied by the RSC was calculated. The percent astrocytosis was defined as: (the fraction of RSC occupied by GFAP-reactive astrocytes) X 100. Similarly, percent microgliosis was defined as: (the fraction of the RSC occupied by MAC-1- or MHC II-reactive microglia) X 100. For all image analyses, six sections at the level of the dorsal hippocampus, each separated by consecutive 240 μm intervals, were quantitated for each animal. In all cases, the treatment status of the animals was unknown to the observer.

Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims. All publications and patent documents cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted.

From the foregoing it will be apparent that the invention provides for a number of uses. For example, the invention provides for the use of any of the antibodies to Aβ described above in the treatment, prophylaxis or diagnosis of amyloidogenic disease, or in the manufacture of a medicament or diagnostic composition for use in the same. Likewise, the invention provides for the use of any of the epitopic fragments of Aβ described above for the treatment or prophylaxis of amyloidogenic disease or in the manufacture of a medicament for use in the same.

TABLE 1

TITER AT 50% MAXIMAL O.D.

Aggreated Aβ Injected mice

Age of PDAPP	mouse 100	mouse 101	mouse 102	mouse 103	mouse 104	mouse 105	mouse 106	mouse 107	mouse 108
4	70000	150000	15000	120000	1000	15000	50000	80000	100000
6	15000	65000	30000	55000	300	15000	15000	50000	60000
8	20000	55000	50000	50000	400	15000	18000	50000	60000
10	40000	20000	60000	50000	900	15000	50000	20000	40000
12	25000	30000	60000	40000	2700	20000	70000	25000	20000

PBS Injected mice on both Immunogens

at 1/100

Age of PDAPP	mouse 113	mouse 114	mouse115	mouse116	mouse117
6	< 4x bkg	< 4x bkg	< 4x bkg	< 4x bkg	< 4x bkg
10	5 x bkg	< 4x bkg	< 4x bkg	< 4x bkg	< 4x bkg
12	< 4x bkg	< 4x bkg	< 4x bkg	< 4x bkg	< 4x bkg

WHAT IS CLAIMED IS:

1. A method of preventing or treating a disease associated with amyloid deposits of aβ in the brain of a patient, comprising administering an effective dosage of an antibody that binds to aβ to the patient.

2. The method of claim 1, wherein the disease is characterized by cognitive impairment.

3. The method of claim 1, wherein the disease is Alzheimer's disease.

4. The method of claim 1, wherein the disease is Down's syndrome.

5. The method of claim 1, wherein the disease is mild cognitive impairment.

6. The method of claim 1, wherein the antibody is of human isotype IgG1.

7. The method of any of the preceding claims, wherein the patient is human.

8. The method of any of the preceding claims, wherein the antibody specifically binds to an epitope within residues 1-10 of Aβ.

9. The method of any of claims 1-8 , wherein the antibody specifically binds to an epitope within residues 1-6 of Aβ.

10. The method of any of claims 1-8, wherein the antibody specifically binds to an epitope within residues 1-5 of Aβ.

11. The method of any of claims 1-8, wherein the antibody specifically binds to an epitope within residues 1-7 of Aβ.

12. The method of any of claims 1-8, wherein the antibody specifically binds to an epitope within residues 3-7 of Aβ.

13. The method of any of claims 1-8, wherein the antibody specifically binds to an epitope within residues 1-3 of Aβ.

14. The method of any of claims 1-8, wherein the antibody specifically binds to an epitope within residues 1-4 of Aβ.

15. The method of any of the preceding claims, wherein after administration the antibody binds to an amyloid deposit in the patient and induces a clearing response against the amyloid deposit.

16. The method of claim 15, wherein the clearing response is an Fc receptor mediated phagocytosis response.

17. The method of claim 15 or 16, further comprising monitoring the clearing response.

18. The method of any of the preceding claims, wherein the antibody specifically binds to an epitope comprising a free N-terminal residue of Aβ.

19. The method of any of the preceding claims, where in the antibody binds to an epitope within residues of 1-10 of Aß wherein residue 1 and/or residue 7 of Aβ is iso-aspartic acid.

20. The method of any of the preceding claims, wherein the patient is asymptomatic.

21. The method of any of the preceding claims, wherein the patient is under 50.

22. The method of any of the preceding claims, wherein the patient has an inherited risk factor indicating susceptibility to Alzheimer's disease.

23. The method of any of claims 1-22, wherein the patient has no known risk factors for Alzheimer's disease.

24. The method of any of the preceding claims, wherein the antibody is a human antibody.

25. The method of any of claims 1-23, wherein the antibody is a humanized antibody.

26. The method of any of claims 1-23, wherein the antibody is a chimeric antibody.

27. The method of any of claims 1-23, wherein the antibody is a mouse antibody.

28. The method of any of the preceding claims, wherein the antibody is a polyclonal antibody.

29. The method of any of claims 1-27, wherein the antibody is a monoclonal antibody.

30. The method of any of the preceding claims, further comprising administering an effective dosage of at least one other antibody that binds to a different epitope of Aβ.

31. The method of claims 1-5 or claims 7-30, wherein the isotype of the antibody is IgG1 or IgG4.

32. The method of any of claims 1-5 or 7-30, wherein the isotype of the antibody is IgG2 or IgG3.

33. The method of any of claims 1-32, wherein the antibody comprises two copies of the same pair of light and heavy chains.

34. The method of any of claims 1-32, wherein the antibody is a bispecific antibody comprising a first light and heavy chain pair that specifically binds to the epitope of Aβ and a second light and heavy chain pair that specifically binds to an Fc receptor on microglial cells.

35. The method of any of the preceding claims, wherein a chain of the antibody is fused to a heterologous polypeptide.

36. The method of any of the preceding claims, wherein the dosage of antibody is at least 1 mg/kg body weight of the patient.

37. The method of claim 36, wherein the dosage of antibody is at least 10 mg/kg body weight of the patient.

38. The method of any of the preceding claims, wherein the antibody is administered with a carrier as a pharmaceutical composition.

39. The method of claims 1-24, 28 and 29-38, wherein the antibody is a human antibody to Aβ prepared from B cells from a human immunized with an Aβ peptide.

40. The method of claim 39, wherein the human immunized with Aβ peptide is the patient.

41. The method of any of the preceding claims, wherein the antibody specifically binds to Aβ peptide without binding to full-length amyloid precursor protein (APP).

42. The method of any of the preceding claims, wherein the antibody is administered intraperitoneally, orally, intranasally, subcutaneously, intramuscularly, topically or intravenously.

43. The method of any of the preceding claims, wherein the antibody is administered by administering a polynucleotide encoding at least one antibody chain to the patient, wherein the polynucleotide is expressed to produce the antibody chain in the patient.

44. The method of claim 43, wherein the polynucleotide encodes heavy and light chains of the antibody, which polynucleotide is expressed to produce the heavy and light chains in the patient.

45. The method of any of the preceding claims, further comprising monitoring the patient for level of administered antibody in the blood of the patient.

46. The method of any of the preceding claims, wherein the antibody is administered in multiple dosages over a period of at least six months.

47. The method of any of claims 1-45, wherein the antibody is administered as a sustained release composition.

48. A method of preventing or treating a disease associated with amyloid deposits of Aβ in the brain of a patient, comprising administering to the patient an effective dosage of a polypeptide comprising an N-terminal segment of at least residues 1-5 of Aβ, the first residue of Aβ being the N-terminal residue of the polypeptide, wherein the polypeptide is free of a C-terminal segment of Aβ.

49. The method of claim 48, wherein the disease is characterized by cognitive impairment.

50. The method of claim 48, wherein the disease is Alzheimer's disease.

51. The method of claim 48, wherein the disease is Down's syndrome.

52. The method of claim 48, wherein the disease is mild cognitive impairment.

53. A method of preventing or treating associated with amyloid deposits of Aβ in the brain of a patient, comprising administering to the patient an effective dosage of a polypeptide comprising an N-terminal segment of Aβ, the segment beginning at residue 1-3 of Aβ and ending at residues 7-11 of Aβ.

54. The method of claim 53, wherein the disease is characterized by cognitive impairment.

55. The method of claim 53, wherein the disease is Alzheimer's disease.

56. The method of claim 53, wherein the disease is Down's syndrome.

57. The method of claim 53, wherein the disease is mild cognitive impairment.

58 The method of any of claims 48-57, wherein the N-terminal segment of Aβ is linked at its C-terminus to a heterologous polypeptide.

59. The method of claim 58, wherein the N-terminal segment consists of the amino acid sequence DAEFRHD.

60. The method of claim 59, wherein the polypeptide comprises the amino acid sequence DAEFRHDQYIKANSKFIGITEL.

61. The method of claim 53, wherein the N-terminal segment of Aβ is linked at its N-terminus to a heterologous polypeptide.

62. The method of claim 61, wherein the polypeptide comprises the amino acid sequence AKXVAAWTLKAAADAEFRHD.

63. The method of claim 53, wherein the N-terminal segment of Aβ is linked at its N and C termini to first and second heterologous polypeptides.

64. The method claim 53, wherein the N-terminal segment of Aβ is linked at its N terminus to a heterologous polypeptide, and at its C-terminus to at least one additional copy of the N-terminal segment.

65. The method of any of claims 58-62 and 64, wherein the heterologous polypeptide induces a T-cell response against the heterologous polypeptide and thereby a B-cell response against the N-terminal segment.

66. The method of any of claims 48-62 and 65, wherein the polypeptide further comprises at least one additional copy of the N-terminal segment.

67. The method of claim 53, wherein the polypeptide comprises from N-terminus to C-terminus, the N-terminal segment of Aβ, a plurality of additional copies of the N-terminal segment, and the heterologous amino acid segment.

68. The method of any of claims 48-67, wherein the N-terminal segment consists of Aβ1-7.

69. The method of any of claims 48-58, 61, and 63, wherein the N-terminal segment consists of Aβ3-7.

70. The method of any of claims 48-57, wherein the polypeptide consists of Aβ1-7.

71. The method of any of claims 48-57, wherein the polypeptide consists of Aβ3-7.

71. The method of any of claims 48-67, wherein the polypeptide is free of at least the 5 C-terminal amino acids in Aβ43.

72. The method of any of claims 48-71, wherein the polypeptide is administered with an adjuvant that enhances an immune response to the N-terminal segment.

73. The method of claim 72, wherein the adjuvant and the polypeptide are administered together as a composition.

74. The method of claim 72, wherein the adjuvant is administered before the polypeptide.

75. The method of claim 72, wherein the adjuvant is administered after the polypeptide.

76. The method of any of claims 72-75, wherein the adjuvant is alum.

77. The method of any of claims 72-75, wherein the adjuvant is MPL.

78. The method of any of claims 72-75, wherein the adjuvant is QS-21.

79. The method of any of claims 72-75, wherein the adjuvant is incomplete Freund's adjuvant.

80. The method of any of claims 48-79, wherein the dosage of the polypeptide is greater than 10 micrograms.

81. A method of preventing or treating a disease associated with amyloid deposits of Aβ in the brain of a patient, comprising administering to a patient an effective dosage of an agent that induces an immunogenic response against an N-terminal segment of Aβ, the segment beginning at residue 1-3 of Aβ and ending at residues 7-11 of Aβ without inducing an immunogenic response against an epitope within residues 12-43 of Aβ43.

82. The method of claim 81, wherein the disease is characterized by cognitive impairment.

83. The method of claim 81, wherein the disease is Alzheimer's disease.

84. The method of claim 81, wherein the disease is Down's syndrome.

85. The method of claim 81, wherein the disease is mild cognitive impairment.

86. A pharmaceutical composition comprising a polypeptide as defined in any of claims 48-71 and an adjuvant.

87. A pharmaceutical composition comprising an antibody as defined in any of claims 1-19, 24-29, 31-35, 39 and 40, and a pharmaceutically acceptable carrier.

88. A method of screening an antibody for activity in treating a disease associated with amyloid deposits of Aβ in the brain of a patient, comprising

contacting the antibody with a polypeptide comprising at least five contiguous amino acids of an N-terminal segment of Aβ beginning at a residue between 1 and 3 of Aβ, the polypeptide being free of a C-terminal segment of Aβ,

and determining whether the antibody specifically binds to the polypeptide, specific binding providing an indication that the antibody has activity in treating Alzheimer's disease.

89. The method of claim 88, wherein the disease is Alzheimer's disease.

90. A method of screening an antibody for activity in clearing a biological entity physically associated with an antigen, comprising

combining the antigen-associated biological entity, the antibody and phagocytic cells bearing Fc receptors in a medium;

monitoring the amount of the antigen-associated biological entity remaining in the medium, a reduction in amount of the antigen-associated biological entity indicating the antibody has clearing activity against the antigen.

91. The method of claim 90, wherein the monitoring step monitors the amount of the antigen remaining in the medium.

92. The method of claim 90 or 91, wherein the combining comprises adding antigen-associated biological entity to the medium, and contacting the medium with the phagocytic cells bearing Fc receptors.

93. The method of any of claims 90-92, wherein the antigen-associated biological entity is provided as a tissue sample.

94. The method of any of claims 90-93, wherein the antigen is the biological entity.

95. The method of claim 94, wherein the tissue sample comprises an amyloid deposit.

96. The method of any of claims 93-95, wherein the tissue sample is from the brain of an Alzheimer's disease patient or a mammal having Alzheimer's pathology.

97. The method of any of claims 90-96, wherein the antigen is Aβ.

98. The method of any of claims 90-97, wherein the phagocytic cells are microglial cells.

99. The method of any of claims 93-95, wherein the tissue sample is selected from the group consisting of a cancerous tissue sample, a virally infected tissue sample, a tissue sample comprising inflammatory cells, a nonmalignant abnormal cell growth, and a tissue sample comprising an abnormal extracellular matrix.

100. A method of detecting an amyloid deposit in a patient, comprising administering to the patient an antibody that specifically binds to an epitope within amino acids 1-10 of Aβ;

detecting presence of the antibody in the brain of the patient.

101. The method of claim 100, wherein the antibody binds to an epitope within residues 4-10 of Aβ.

102. The method of claim 100, wherein the antibody binds to an epitope within residues 8-10 of Aβ.

103. The method of any of claims 100-102, wherein the antibody is labelled.

104. The method of claim 103, wherein the antibody is labelled with a paramagnetic label.

105. The method of claim 104, wherein the labelled antibody is detected by nuclear magnetic resonance.

106. The method of any of claims 100-105, wherein the antibody lacks capacity to induce a clearance response on binding to an amyloid deposit in the patient.

107. A diagnostic kit, comprising

an antibody that specifically binds to an epitope with residues 1-10 of Aβ.

108. The kit of claim 107, further comprising labeling describing use of the antibody for in vivo diagnosis or monitoring of a disease associated with amyloid deposits of Aβ in the brain of a patient.

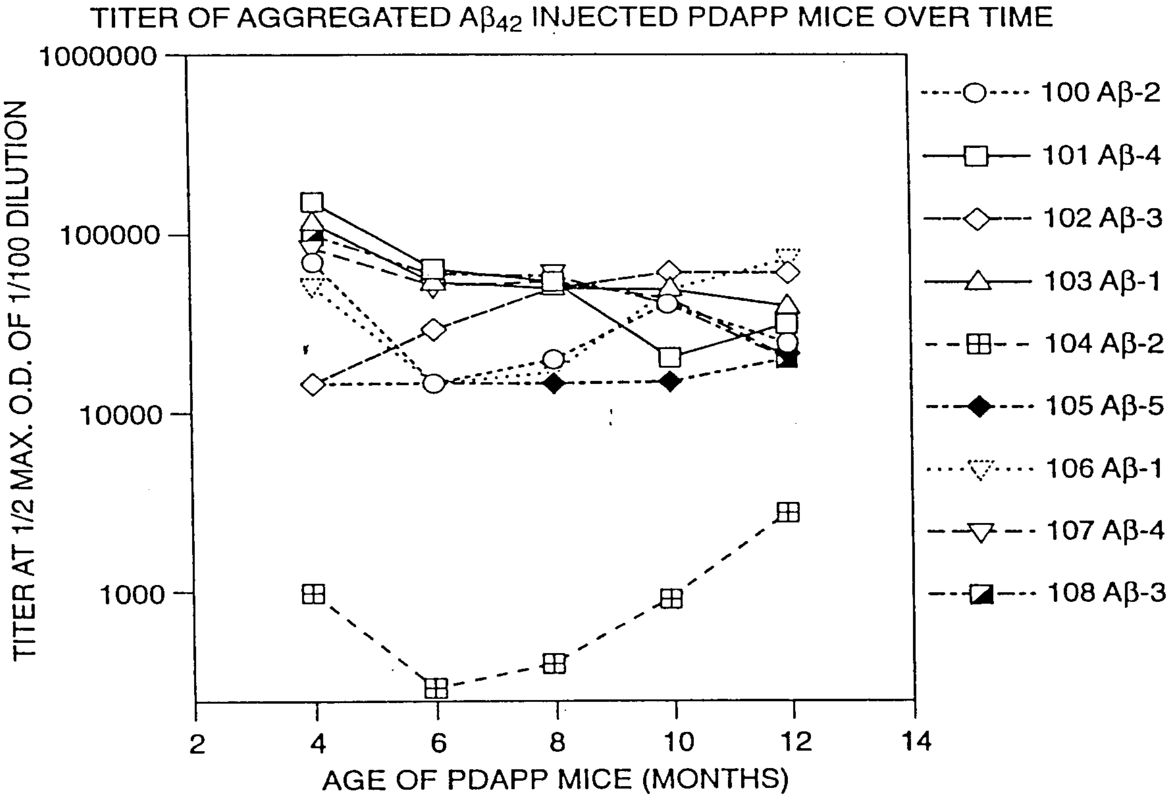

TITER OF AGGREGATED Aβ42 INJECTED PDAPP MICE OVER TIME
TITER AT 1/2 MAX. O.D. OF 1/100 DILUTION
1000000
100000
10000
1000
AGE OF PDAPP MICE (MONTHS)
2
4
6
8
10
12
14
100 Aβ-2
101 Aβ-4
102 Aβ-3
103 Aβ-1
104 Aβ-2
105 Aβ-5
106 Aβ-1
107 Aβ-4
108 Aβ-3

FIG. 1

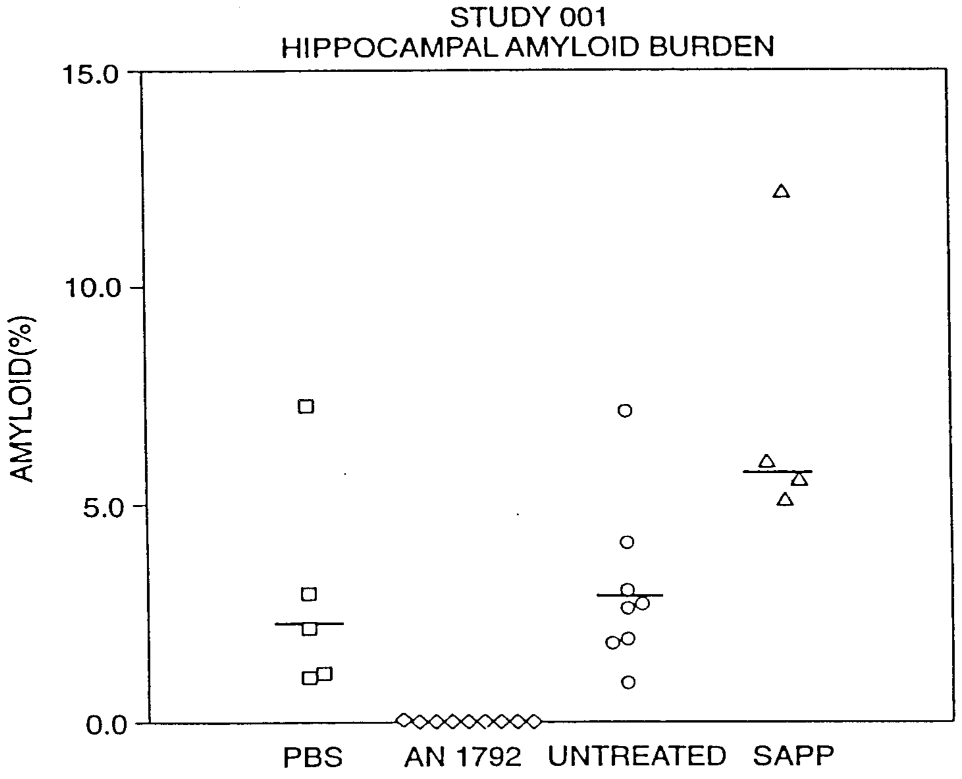

STUDY 001
HIPPOCAMPAL AMYLOID BURDEN
AMYLOID(%)
15.0
10.0
5.0
0.0
PBS
AN 1792
UNTREATED
SAPP

FIG. 2

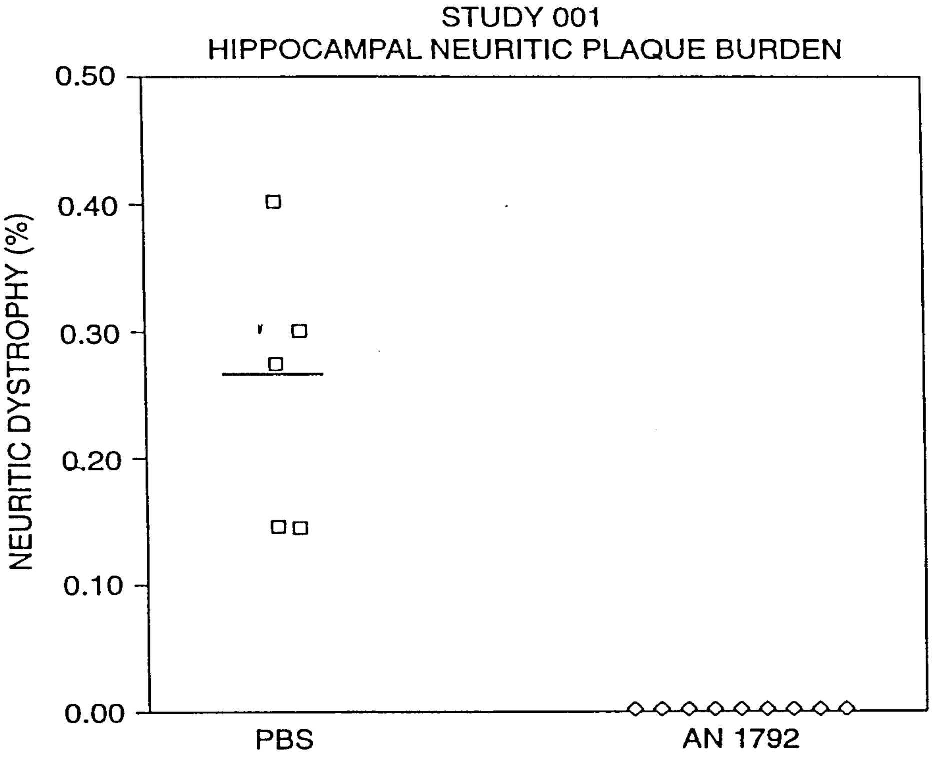

STUDY 001
HIPPOCAMPAL NEURITIC PLAQUE BURDEN
NEURITIC DYSTROPHY (%)
0.50
0.40
0.30
0.20
0.10
0.00
PBS
AN 1792

FIG. 3

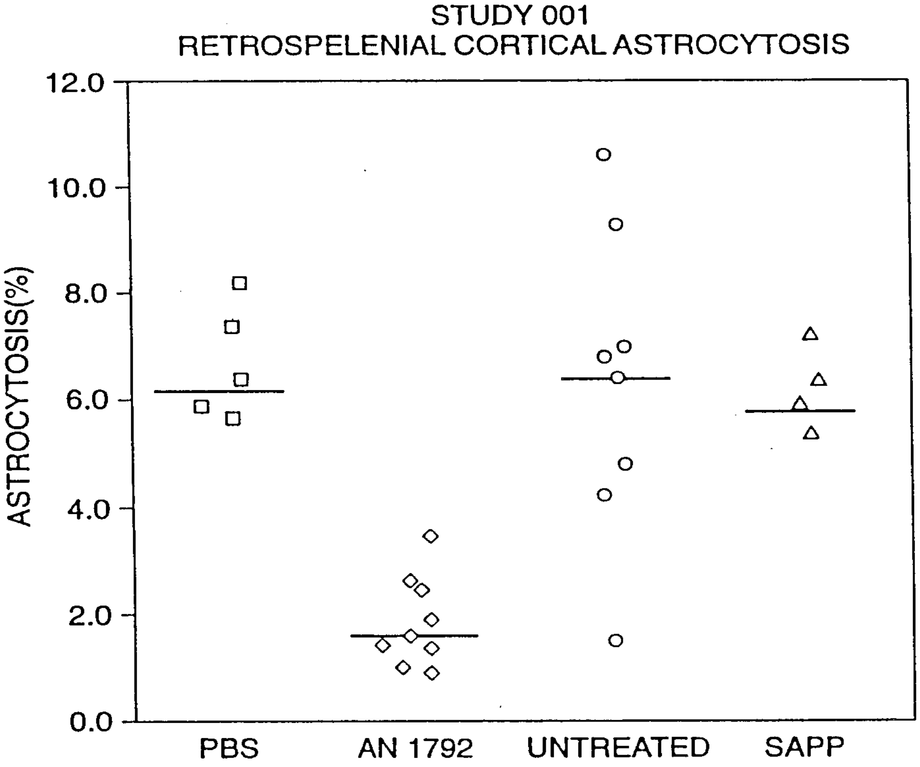

STUDY 001
RETROSPELENIAL CORTICAL ASTROCYTOSIS
ASTROCYTOSIS(%)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
PBS
AN 1792
UNTREATED
SAPP

FIG. 4

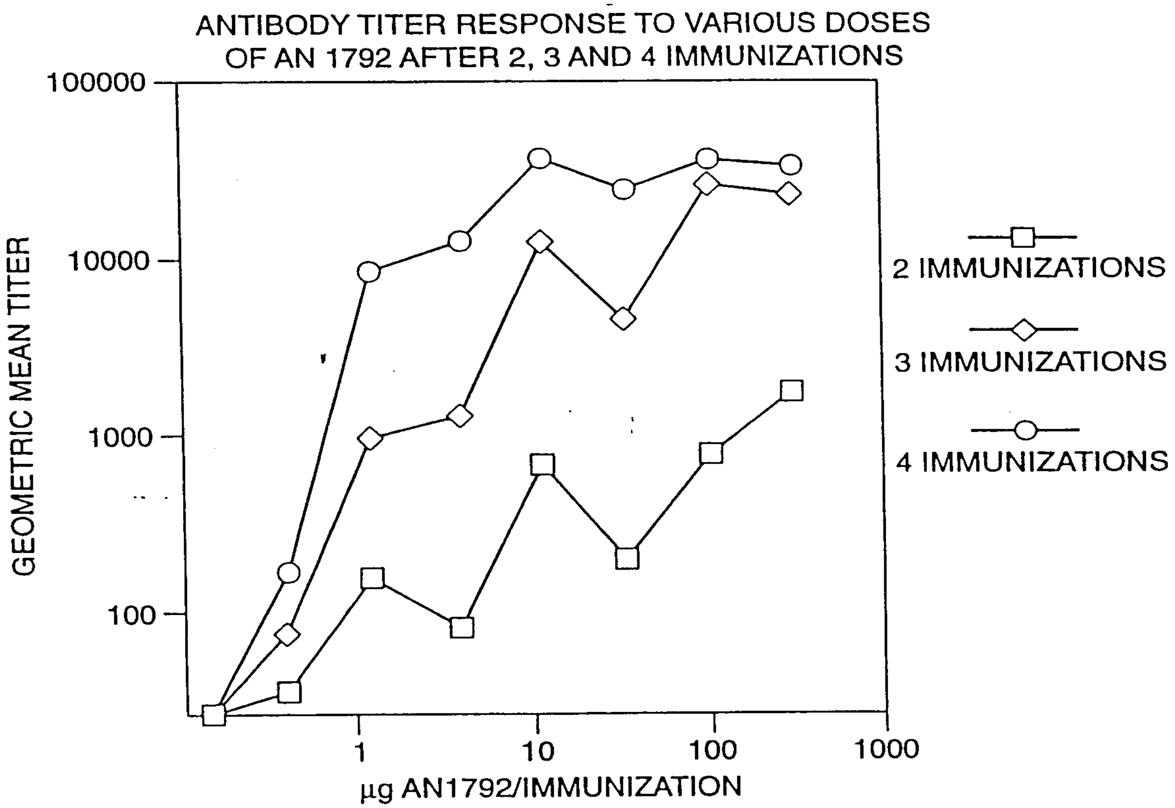

ANTIBODY TITER RESPONSE TO VARIOUS DOSES OF AN 1792 AFTER 2, 3 AND 4 IMMUNIZATIONS
GEOMETRIC MEAN TITER
100000
10000
1000
100
1
10
100
1000
μg AN1792/IMMUNIZATION
2 IMMUNIZATIONS
3 IMMUNIZATIONS
4 IMMUNIZATIONS

FIG. 5

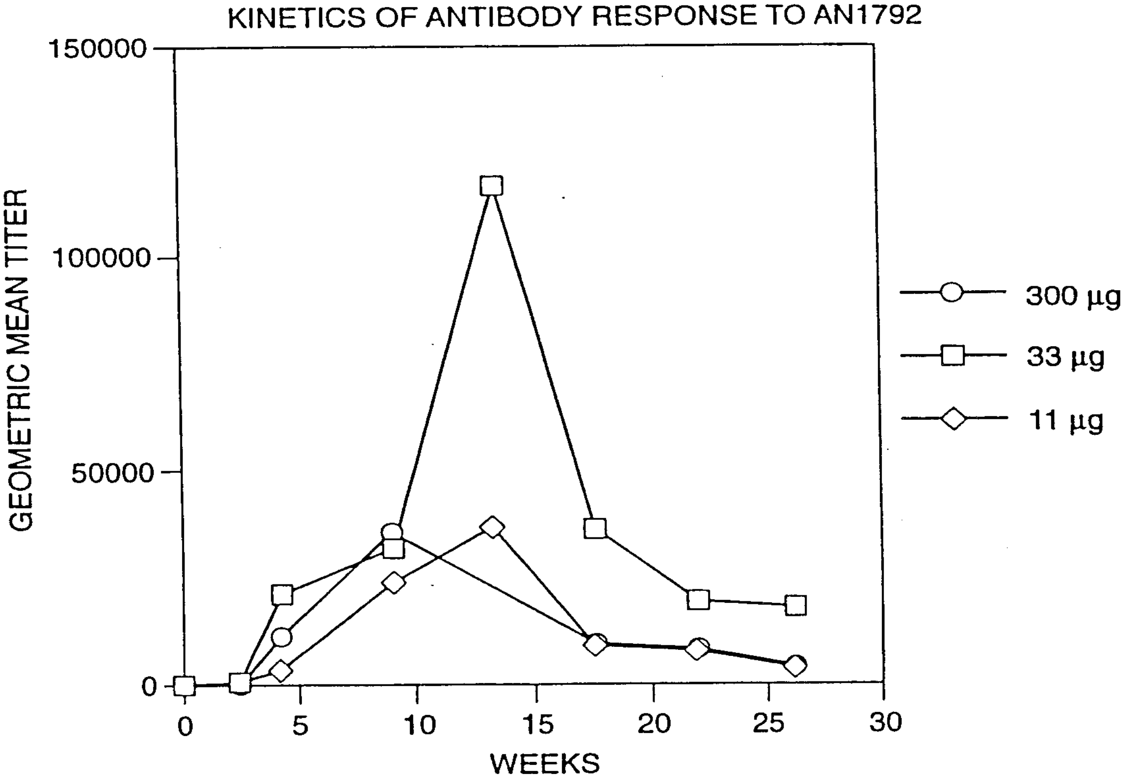

KINETICS OF ANTIBODY RESPONSE TO AN1792
GEOMETRIC MEAN TITER
150000
100000
50000
0
0
5
10
15
20
25
30
WEEKS
300 μg
33 μg
11 μg

FIG. 6

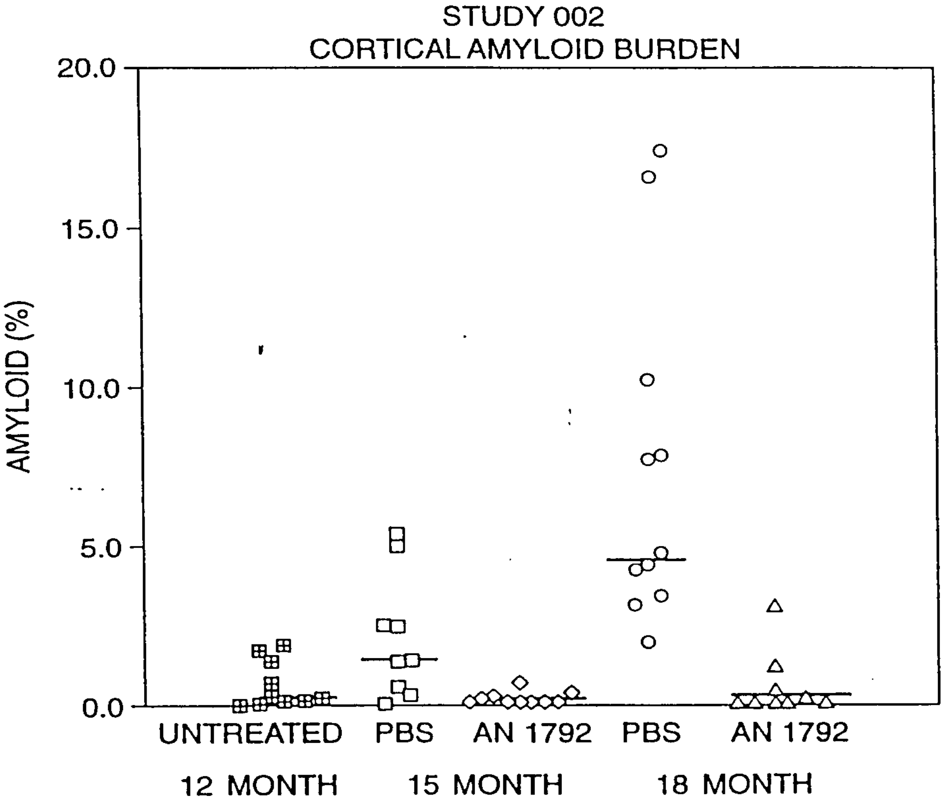

STUDY 002
CORTICAL AMYLOID BURDEN
20.0
15.0
10.0
5.0
0.0
AMYLOID (%)
UNTREATED
PBS
AN 1792
PBS
AN 1792
12 MONTH
15 MONTH
18 MONTH

FIG. 7

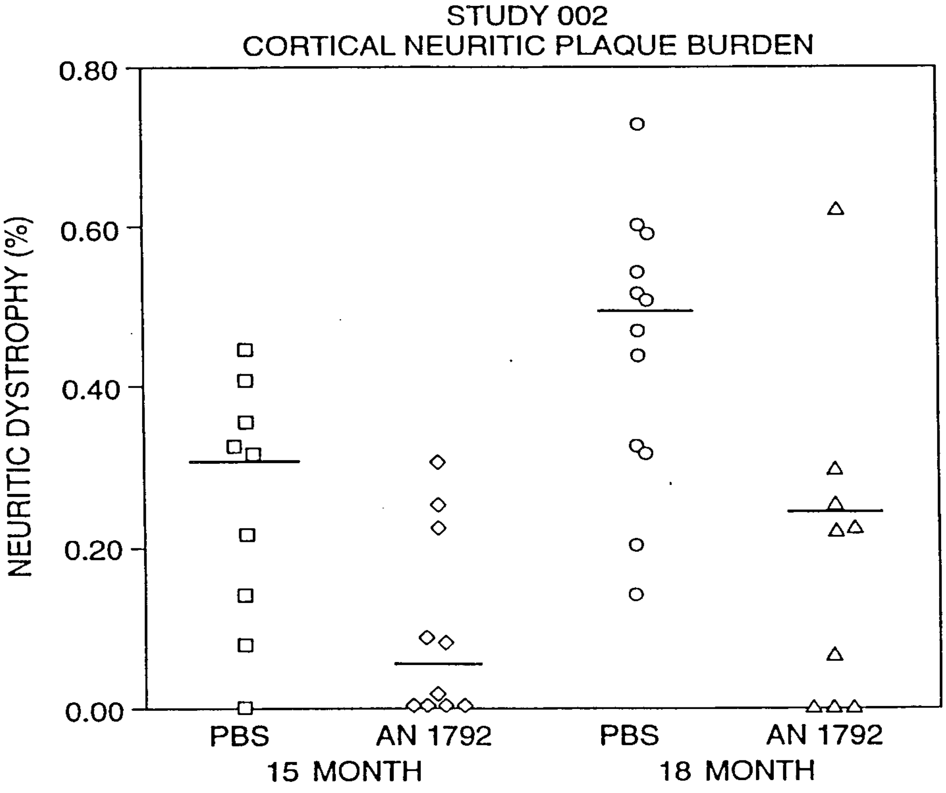

STUDY 002
CORTICAL NEURITIC PLAQUE BURDEN
0.80
0.60
0.40
0.20
0.00
NEURITIC DYSTROPHY (%)
PBS
AN 1792
PBS
AN 1792
15 MONTH
18 MONTH

FIG. 8

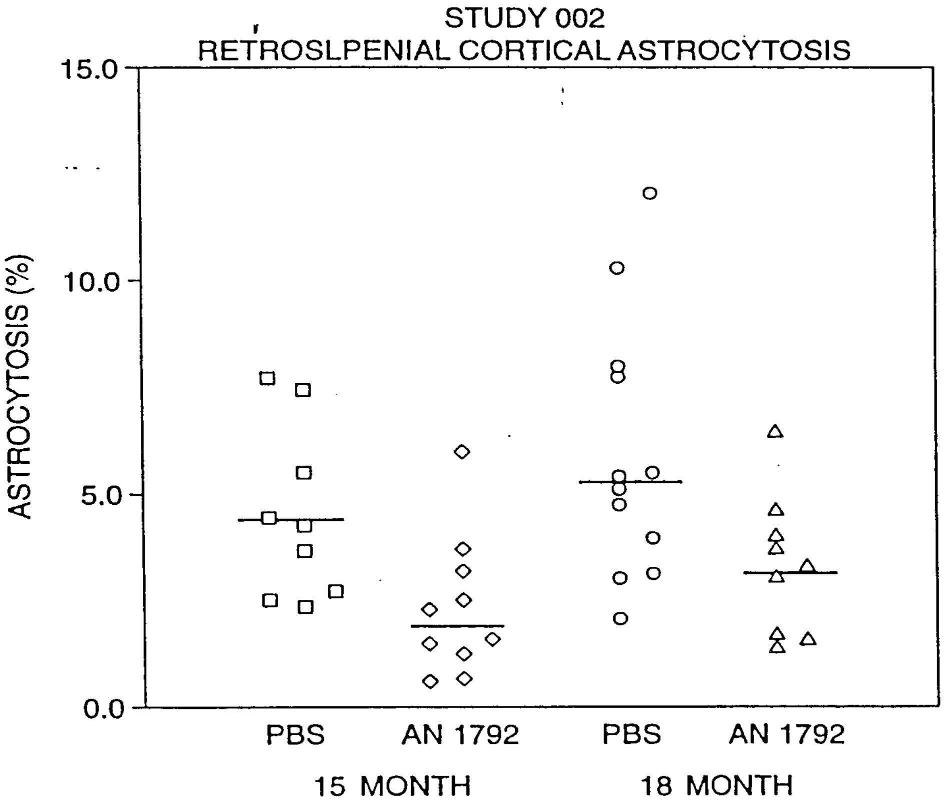

STUDY 002
RETROSLPENIAL CORTICAL ASTROCYTOSIS
ASTROCYTOSIS (%)
15.0
10.0
5.0
0.0
PBS
AN 1792
PBS
AN 1792
15 MONTH
18 MONTH

FIG. 9

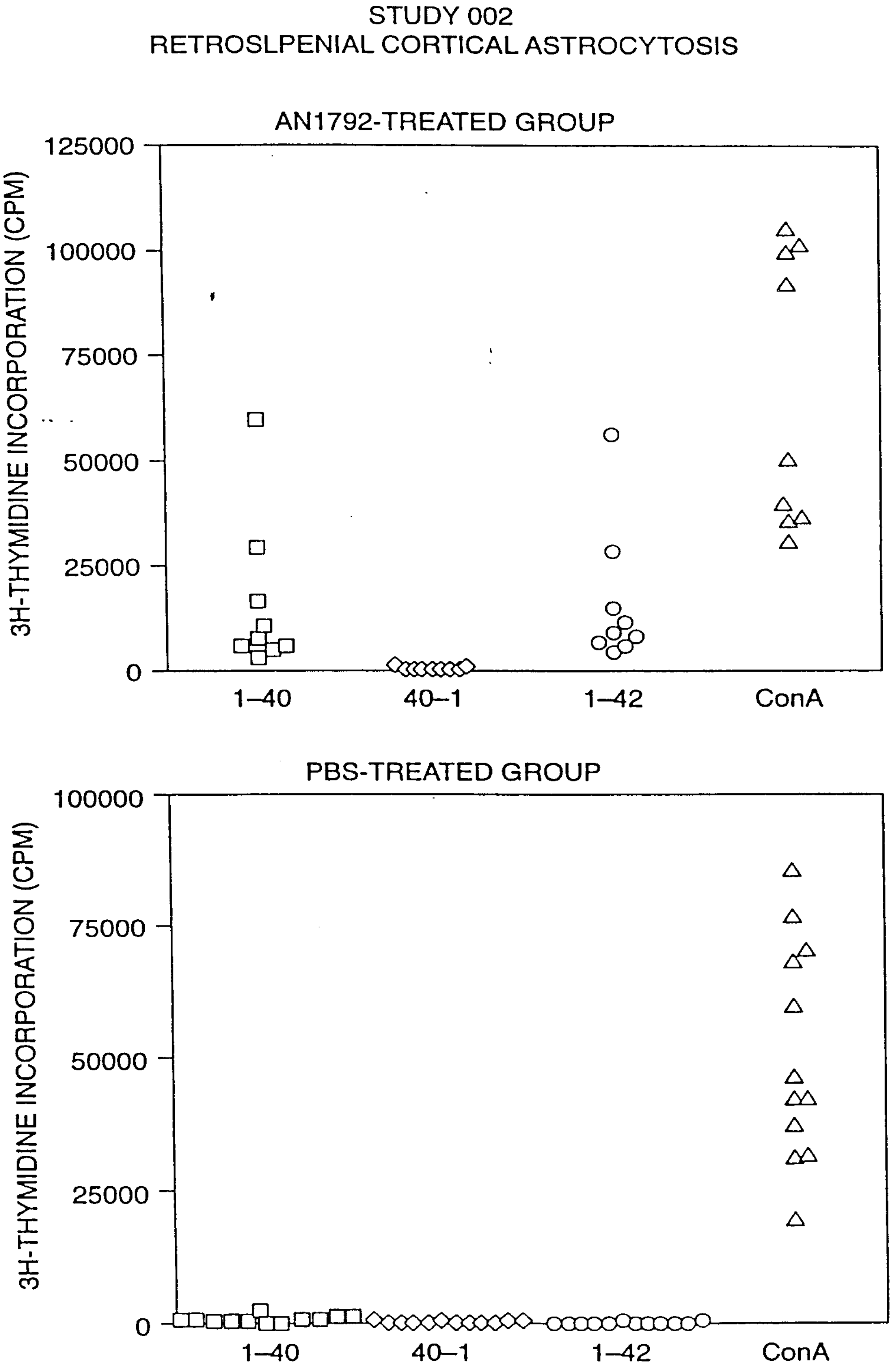

STUDY 002
RETROSLPENIAL CORTICAL ASTROCYTOSIS
AN1792-TREATED GROUP
3H-THYMIDINE INCORPORATION (CPM)
125000
100000
75000
50000
25000
0
1–40
40–1
1–42
ConA
PBS-TREATED GROUP
3H-THYMIDINE INCORPORATION (CPM)
100000
75000
50000
25000
0
1–40
40–1
1–42
ConA

FIG. 10

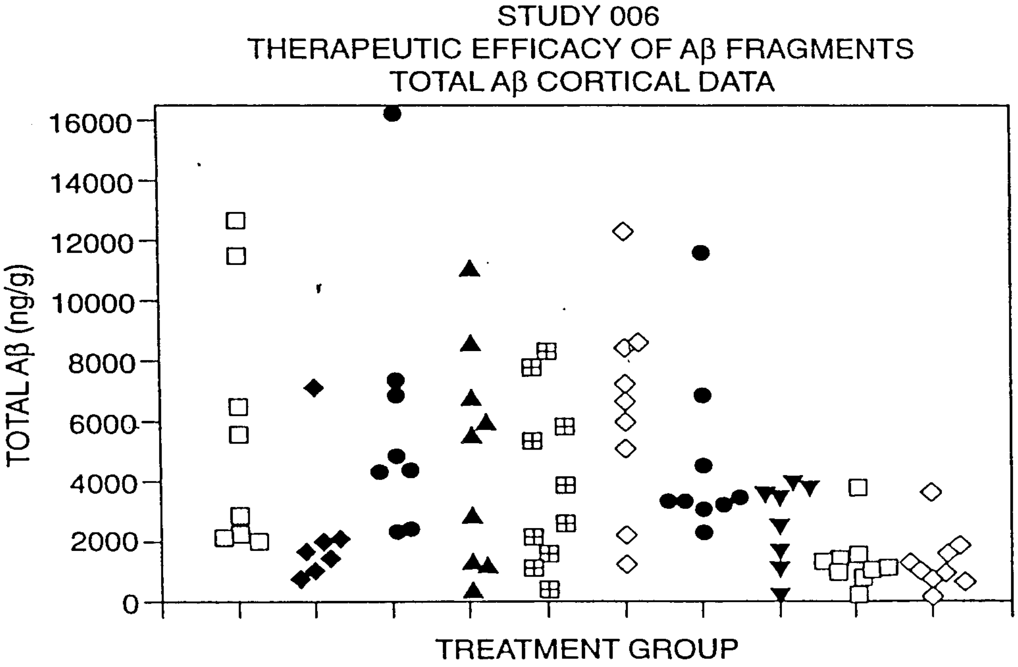

STUDY 006
THERAPEUTIC EFFICACY OF Aβ FRAGMENTS
TOTAL Aβ CORTICAL DATA
16000
14000
12000
10000
8000
6000
4000
2000
0
TOTAL Aβ (ng/g)
TREATMENT GROUP

FIG. 11

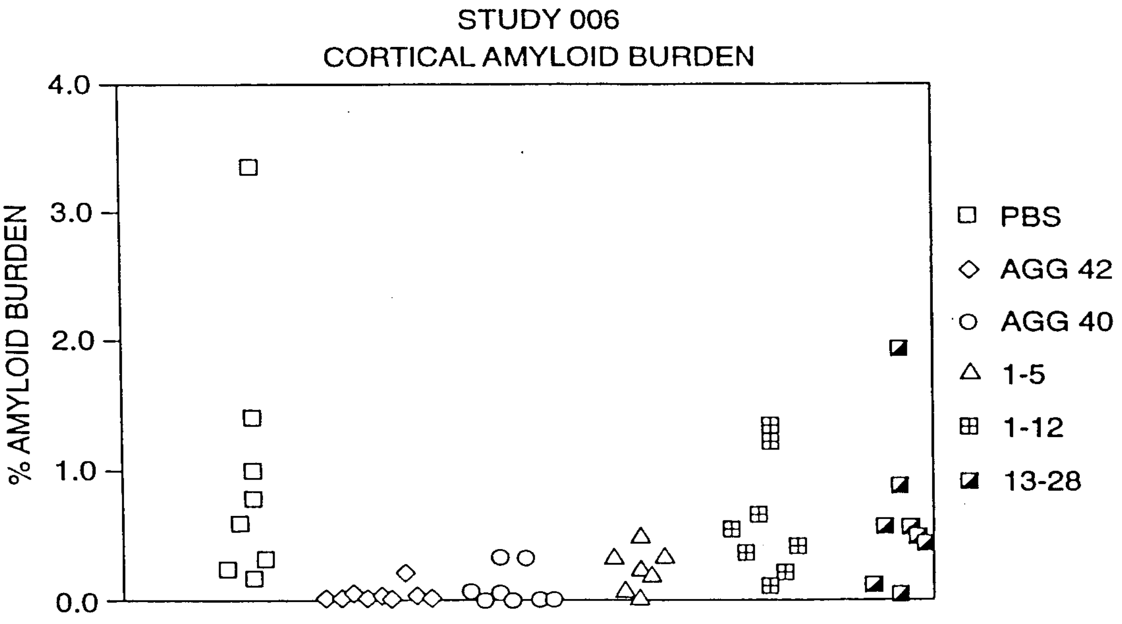

STUDY 006
CORTICAL AMYLOID BURDEN
4.0
3.0
2.0
1.0
0.0
% AMYLOID BURDEN
□ PBS
◇ AGG 42
○ AGG 40
△ 1-5
⊞ 1-12
◪ 13-28

FIG. 12

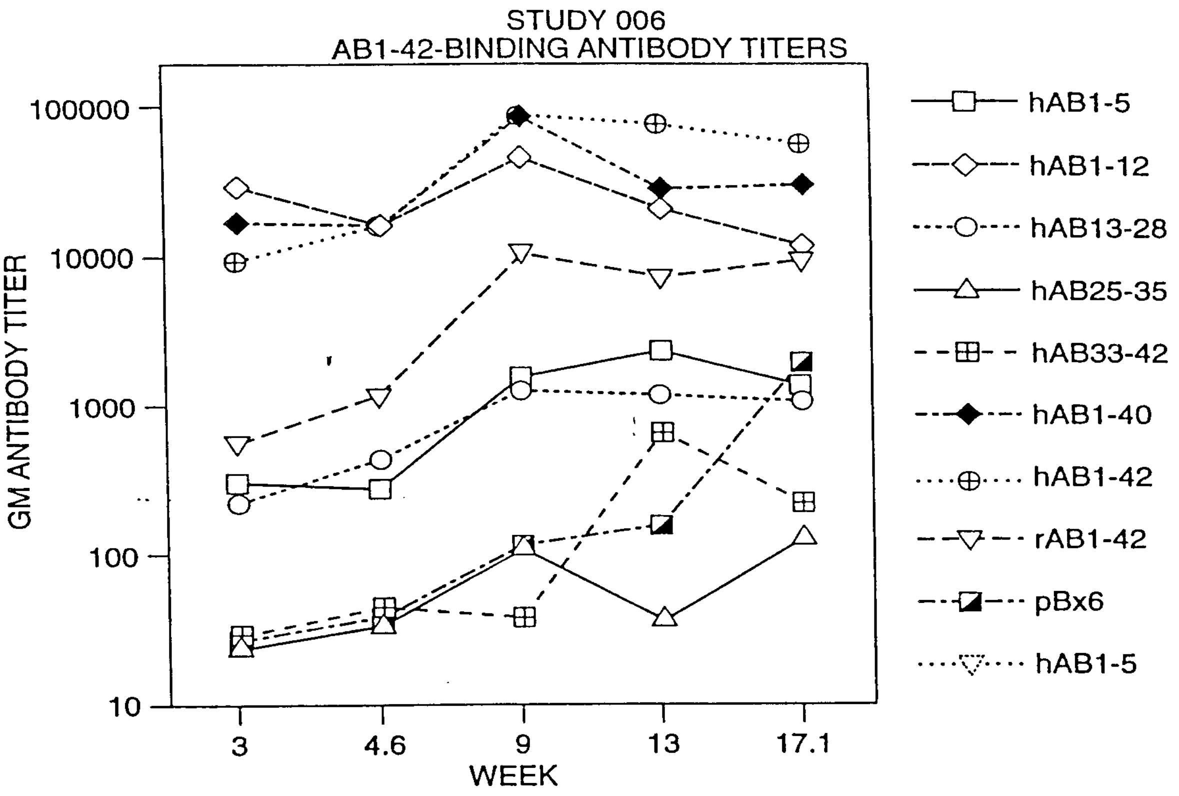

STUDY 006
AB1-42-BINDING ANTIBODY TITERS
GM ANTIBODY TITER
100000
10000
1000
100
10
3
4.6
9
13
17.1
WEEK
hAB1-5
hAB1-12
hAB13-28
hAB25-35
hAB33-42
hAB1-40
hAB1-42
rAB1-42
pBx6
hAB1-5

FIG. 13

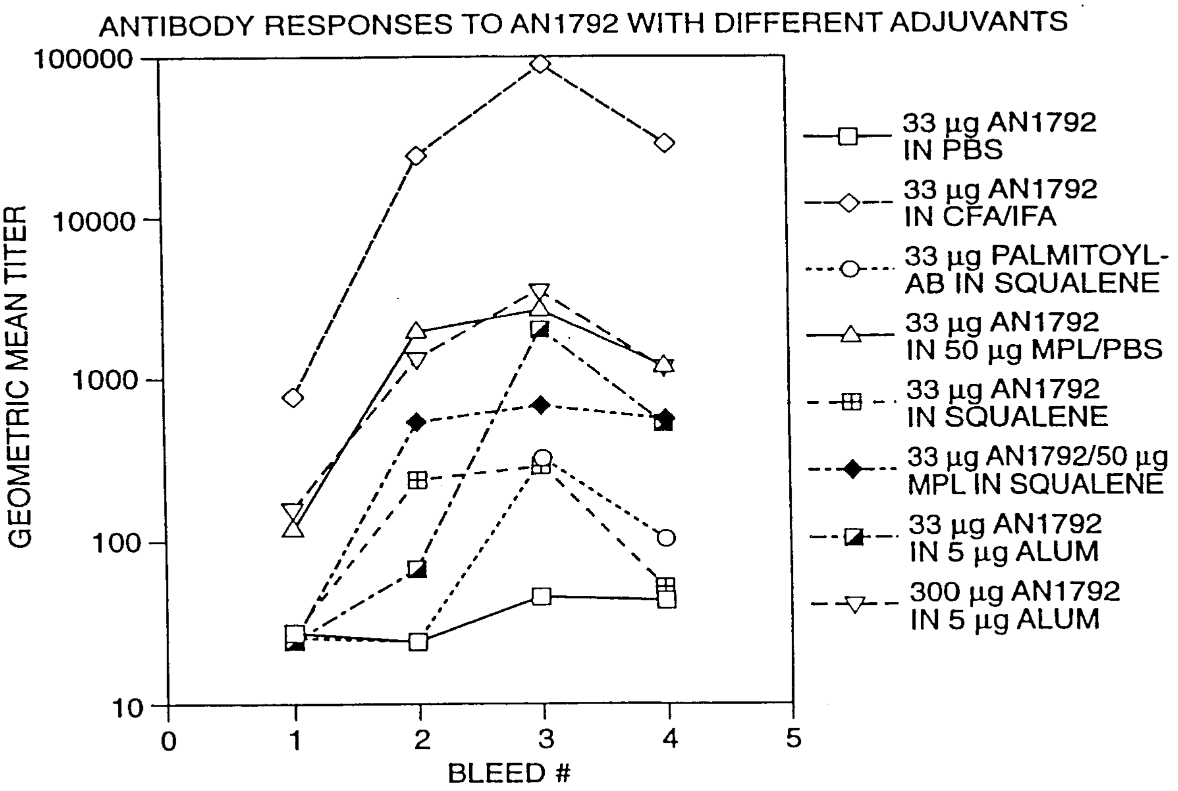

ANTIBODY RESPONSES TO AN1792 WITH DIFFERENT ADJUVANTS
GEOMETRIC MEAN TITER
100000
10000
1000
100
10
0
1
2
3
4
5
BLEED #
33 µg AN1792 IN PBS
33 µg AN1792 IN CFA/IFA
33 µg PALMITOYL-AB IN SQUALENE
33 µg AN1792 IN 50 µg MPL/PBS
33 µg AN1792 IN SQUALENE
33 µg AN1792/50 µg MPL IN SQUALENE
33 µg AN1792 IN 5 µg ALUM
300 µg AN1792 IN 5 µg ALUM

FIG. 14

CORTEX

PBS CONTROL		UNTREATED CONTROL	
624-165	272	764-181	3470
625-166	1802	785-182	171
626-167	62	766-183	91
633-168	4696	767-184	6692
634-169	3090	768-185	1353
671-170	2417	771-186	1153
672-171	2840	772-187	3800
829-172	3320	780-188	3740
830-173	1833	843-189	163
831-174	416	844-190	122
792-175	126	845-191	427
793-176	2559	846-192	2674
794-177	289	887-193	453
732-178	179	888-194	2996
733-179	1329	889-195	1075
734-180	5665		
MEDIAN	1817	MEDIAN	1153
p MALUE (M–W)		p MALUE (M–W)	
MEAN	1931	MEAN	1825
ST. DEV.	1718	ST. DEV.	1769
% CV	89	% CV	97
p VALUE (t TEST)		p VALUE (t TEST)	
	n=16		n=15

FIG. 15A

CORTEX

2 mg ALUM 100 µg AN1528		50 µg MPL 100 µg AN1528	
660-083	295	643-105	385
661-084	3180	644-106	2640
662-085	2480	645-107	2403
663-086	3014	654-108	1741
664-087	5870	655-109	3053
665-088	5978	656-110	5990
693-089	1620	678-111	3360
694-090	35	679-112	1230
695-091	3400	704-114	2680
697-092	2630	705-115	78
698-093	983	706-116	1290
699-094	5327	729-117	3180
701-095	1862	730-118	1833
702-096	1849	731-119	4590
703-097	2239	736-120	1112
739-098	806	737-121	1653
740-099	5303	757-122	992
741-100	459	758-123	4692
800-103	154	808-124	785
801-104	852	809-125	244
		810-126	32
MEDIAN	2051	MEDIAN	1741
p MALUE (M–W)		p MALUE (M–W)	
MEAN	2407	MEAN	2140
ST. DEV.	1913	ST. DEV.	1659
% CV	79	% CV	78
p VALUE (t TEST)		p VALUE (t TEST)	
	n=20		n=21

FIG. 15B

CORTEX

25 µg QS21 100 µg AN1528		CEA/IFA 100 µg AN1792	
615-128	1257	539-068	693
616-129	361	640-069	508
617-130	1008	641-070	440
536-131	3290	642-071	467
637-132	2520	690-072	42
638-133	3880	691-073	2491
744-134	627	692-074	121
745-135	58	795-075	137
746-136	2610	796-076	822
747-137	1509	797-077	475
769-138	1788	748-087	600
770-139	988	749-079	78
773-140	1199	750-080	1267
774-141	339	751-081	1351
775-142	402	761-082	69
776-143	537		
840-144	1119		
841-145	194		
821-146	1259		
822-147	5413		
823-148	2233		
MEDIAN	1199	MEDIAN	475
p MALUE (M–W)		p MALUE (M–W)	0.0481
MEAN	1552	MEAN	637
ST. DEV.	1364	ST. DEV.	655
% CV	88	% CV	103
p VALUE (t TEST)		p VALUE (t TEST)	0.0106
	n=21		n=15

FIG. 15C

CORTEX

5 µg THIMEROSAL/PBS 10 µg AN1792		2 µg ALUM 100 µg AN1792	
635-149	1337	610-001	432
669-150	4644	611-002	1012
670-151	6335	612-003	3607
673-152	3700	613-004	508
674-153	2750	620-005	465
676-154	1687	621-006	16
681-156	185	622-007	28
682-157	8031	623-008	217
683-158	3450	708-009	2738
754-159	157	709-010	927
755-160	6857	710-011	1609
756-161	482	716-012	1608
805-162	524	784-014	3890
806-163	397	785-015	1614
807-164	234	786-018	285
		787-017	3102
		788-018	1617
		789-019	1474
		815-020	424
		816-021	1375
		817-022	2323
MEDIAN	1687	MEDIAN	1375
p MALUE (M–W)		p MALUE (M–W)	0.5000
MEAN	2718	MEAN	1394
ST. DEV.	2685	ST. DEV.	1166
% CV	99	% CV	84
p VALUE (t TEST)		p VALUE (t TEST)	0.2650
	n=15		n=21

FIG. 15D

CORTEX

50 µg MPL 100 µg AN1792		25 µg QS21 100 µg AN1792	
646-023	2002	627-045	91
647-024	147	628-046	3397
648-025	1304	631-049	3702
649-026	34	632-050	1776
650-027	980	667-052	1832
724-028	1282	668-053	3023
726-030	1966	686-054	189
727-031	733	687-055	891
720-032	2563	688-056	240
721-033	5563	689-057	110
802-034	113	712-059	3311
803-035	671	825-061	1009
804-036	51	826-082	18165
811-037	613	827-063	73
812-038	332	828-064	78
813-039	1454	837-065	1051
814-040	2441	838-066	270
833-014	742	839-067	371
834-042	40		
836-044	807		
MEDIAN	774	MEDIAN	950
p MALUE (M–W)	0. 1710	p MALUE (M–W)	0.4076
MEAN	1192	MEAN	2199
ST. DEV.	1299	ST. DEV.	4187
% CV	109	% CV	190
p VALUE (t TEST)	0.1506	p VALUE (t TEST)	0.8131
	n=21		n=18

FIG. 15E

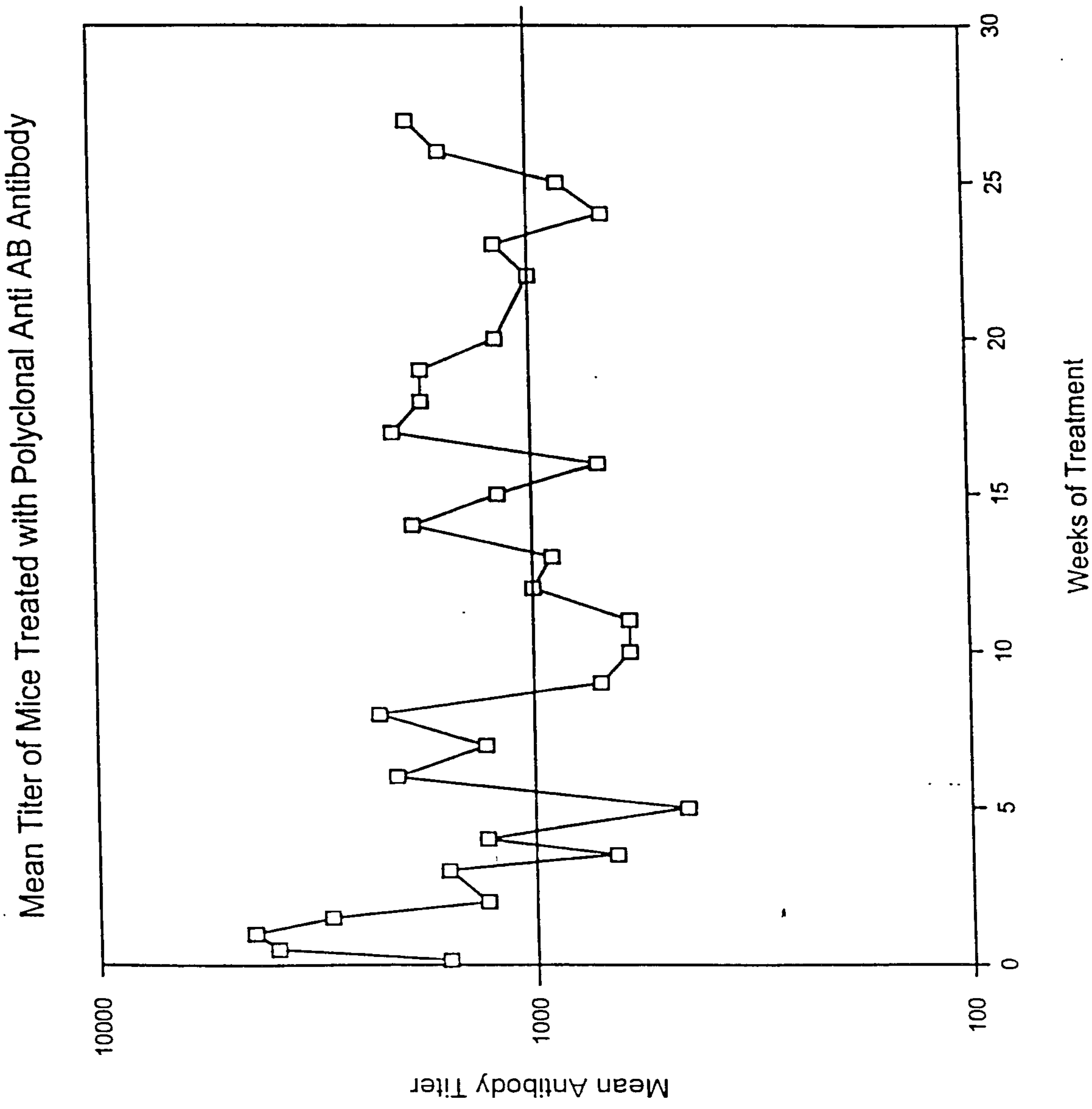

Mean Titer of Mice Treated with Polyclonal Anti AB Antibody
Mean Antibody Titer
10000
1000
100
Weeks of Treatment
0
5
10
15
20
25
30

Figure 16

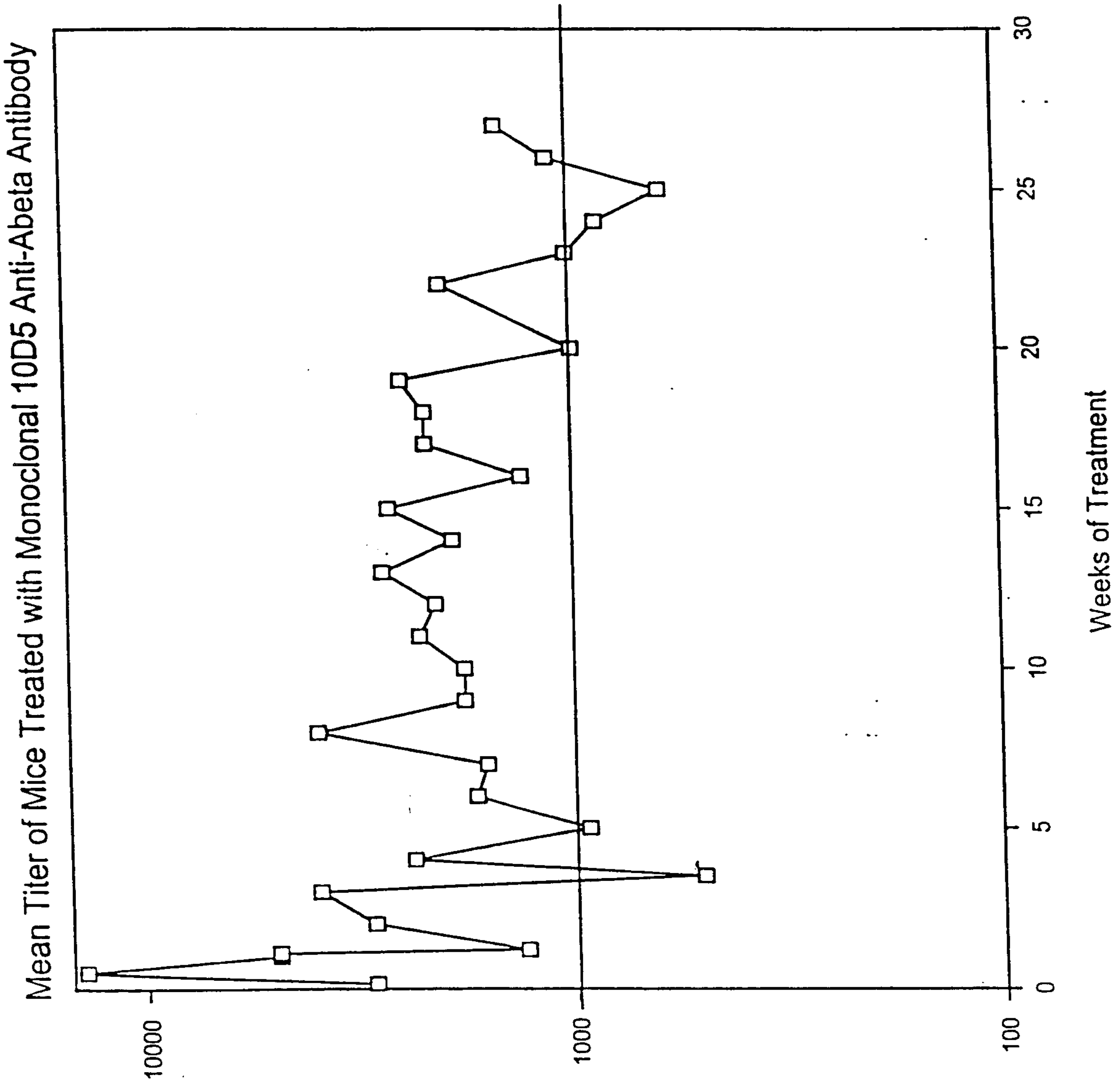

Mean Titer of Mice Treated with Monoclonal 10D5 Anti-Abeta Antibody
Mean Antibody Titer
10000
1000
100
Weeks of Treatment
0
5
10
15
20
25
30

Figure 17

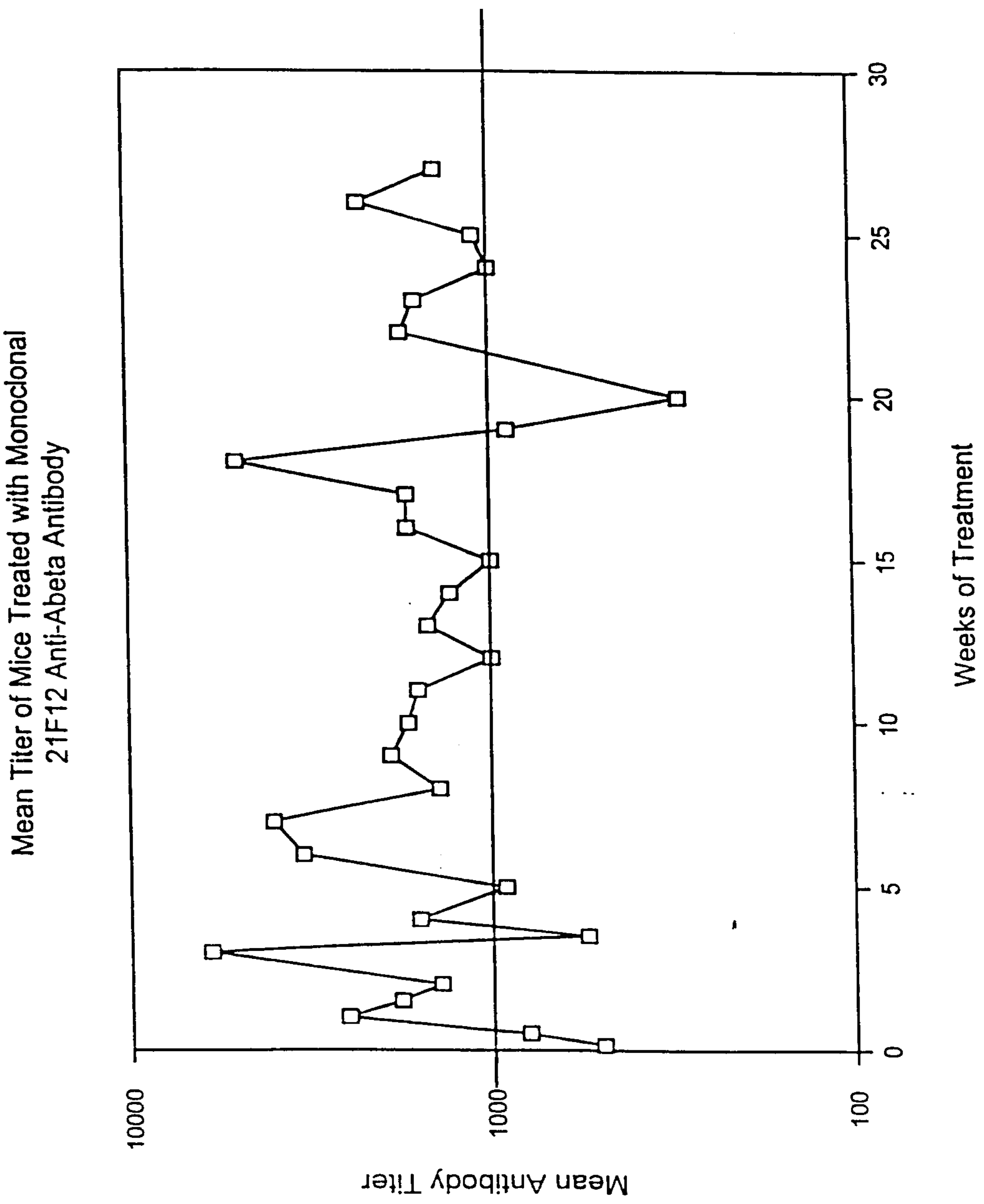

Mean Titer of Mice Treated with Monoclonal
21F12 Anti-Abeta Antibody
Mean Antibody Titer
10000
1000
100
0
5
10
15
20
25
30
Weeks of Treatment

Figure 18

Figure 19:

Group 9: F10920M, 300μg AN1792 + 100μg QS21
Dosing Schedule 2 titer=84,484 (normalized to 8)

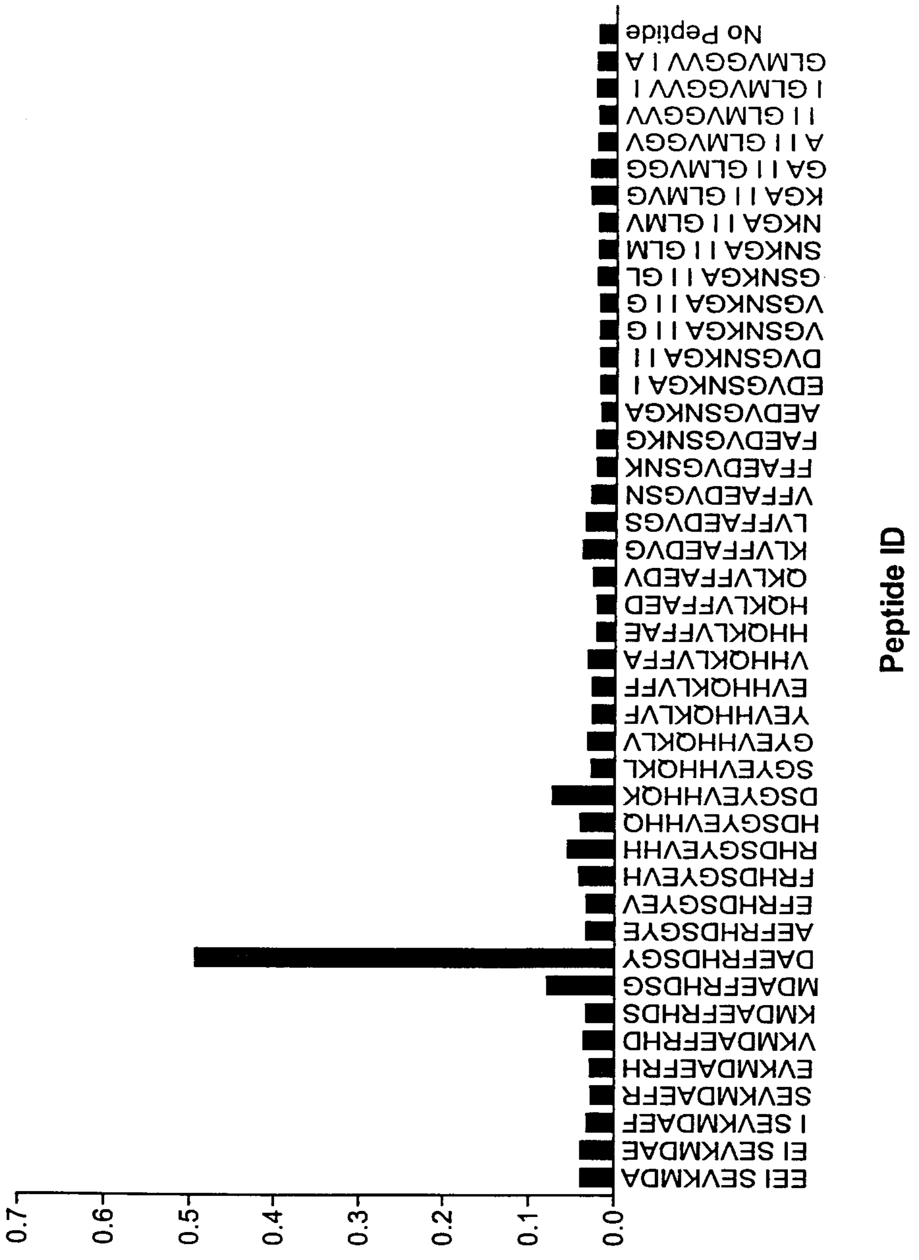
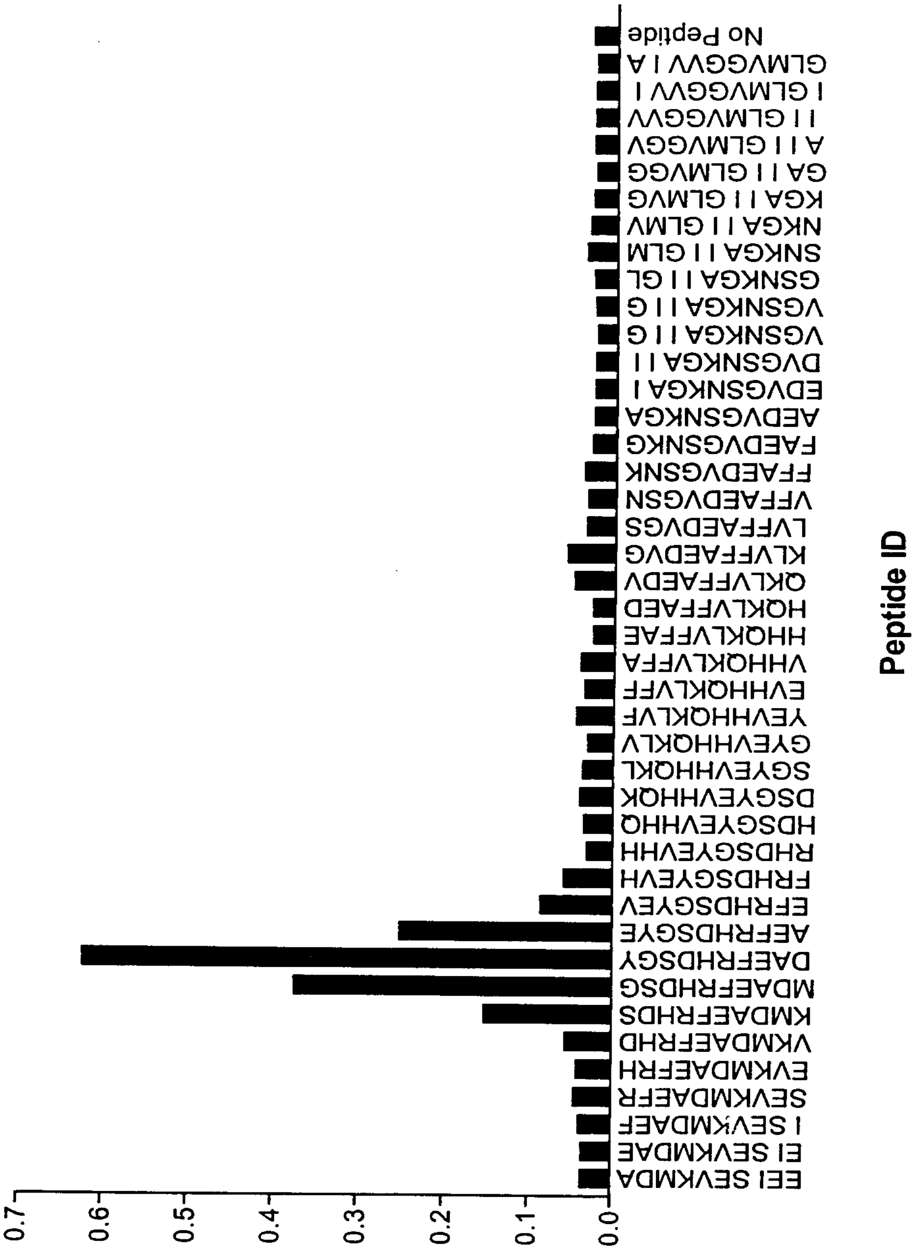


Figure 20:

Group 11: F10975F, 300μg AN1792 + 100μg QS21 Dosing Schedule 3, Titer=38,632 (normalized to 8)



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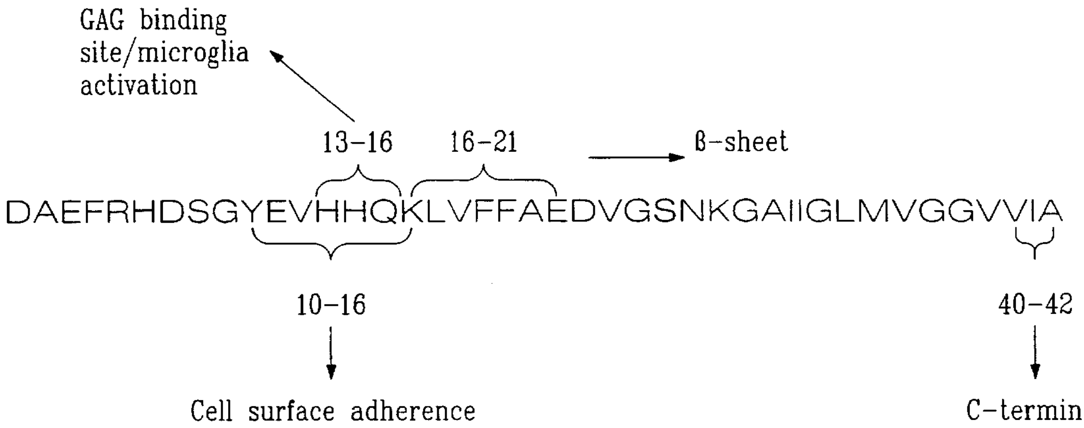
(54) Title: VACCINE FOR THE PREVENTION AND TREATMENT OF ALZHEIMER'S AND AMYLOID RELATED DISEASES


GAG binding site/microglia activation
13-16
16-21
ß-sheet
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
10-16
40-42
Cell surface adherence
C-terminal

(57) Abstract: The present invention relates to a stereochemically based "non-self" antigen vaccine for the prevention and/or treatment of Alzheimer's and other amyloid related diseases. The present invention provides a vaccine for the prevention and treatment of Alzheimer's and other amyloid related diseases, which overcomes the drawbacks associated with using naturally occurring peptides, proteins or immunogens.

WO 01/39796 A2

VACCINE FOR THE PREVENTION AND TREATMENT OF ALZHEIMER'S AND AMYLOID RELATED DISEASES

RELATED APPLICATIONS

This application claims the benefit of priority under 35 U.S.C. 119(e) to copending U.S. Provisional Application No. 60/168,594, filed on November 29, 1999, and under 35 U.S.C. 120 of copending U.S. application No. _________, filed on November 28, 2000, the entire contents of both applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates to a new stereochemically based "non-self" antigen vaccine for the prevention and/or treatment of Alzheimer's and other amyloid related diseases.

Amyloidosis refers to a pathological condition characterized by the presence of amyloid fibers. Amyloid is a generic term referring to a group of diverse but specific protein deposits (intracellular and/or extracellular) which are seen in a number of different diseases. Though diverse in their occurrence, all amyloid deposits have common morphologic properties, stain with specific dyes (e.g., Congo red), and have a characteristic red-green birefringent appearance in polarized light after staining. They also share common ultrastructural features and common x-ray diffraction and infrared spectra.

Amyloid-related diseases can either be restricted to one organ or spread to several organs. The first instance is referred to as “localized amyloidosis” while the second is referred to as “systemic amyloidosis”.

Some amyloidotic diseases can be idiopathic, but most of these diseases appear as a complication of a previously existing disorder. For example, primary amyloidosis can appear without any other pathology or can follow plasma cell dyscrasia or multiple myeloma. Secondary amyloidosis is usually seen associated with chronic infection (such as tuberculosis) or chronic inflammation (such as rheumatoid arthritis). A familial form of secondary amyloidosis is also seen in Familial Mediterranean Fever (FMF). This familial type of amyloidosis, as one of the other types of familial amyloidosis, is genetically inherited and is found in specific population groups. In these two types of amyloidosis, deposits are found in several organs and are thus considered systemic amyloid diseases. Another type of systemic amyloidosis is found in long-term hemodialysis patients. In each of these cases, a different amyloidogenic protein is involved in amyloid deposition.

"Localized amyloidoses" are those that tend to involve a single organ system. Different amyloids are also characterized by the type of protein present in the deposit. For example, neurodegenerative diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease and the like are characterized by the appearance and accumulation of a protease-resistant form of a prion protein (referred to as AScr or PrP-27) in the central nervous system. Similarly, Alzheimer's disease, another neurodegenerative disorder, is characterized by neuritic plaques and neurofibrillary tangles. In this case, the plaque and blood vessel amyloid is formed by the deposition of fibrillar Aβ amyloid protein. Other diseases such as adult-onset diabetes (Type II diabetes) are characterized by the localized accumulation of amyloid in the pancreas.

Once these amyloids have formed, there is no known, widely accepted therapy or treatment which significantly dissolves the deposits *in situ*.

Each amyloidogenic protein has the ability to organize into β-sheets and to form insoluble fibrils which get deposited extracellularly or intracellularly. Each amyloidogenic protein, although different in amino acid sequence, has the same property of forming fibrils and binding to other elements such as proteoglycan, amyloid P and complement component. Moreover, each amyloidogenic protein has amino acid sequences which, although different, will show similarities such as regions with the ability to bind to the glycosaminoglycan (GAG) portion of proteoglycan (referred to as the GAG binding site) as well as other regions which will promote β-sheet formation.

In specific cases, amyloidotic fibrils, once deposited, can become toxic to the surrounding cells. As per example, the Aβ fibrils organized as senile plaques have been shown to be associated with dead neuronal cells and microgliosis in patients with Alzheimer's disease. When tested *in vitro*, Aβ peptide was shown to be capable of triggering an activation process of microglia (brain macrophages), which would explain the presence of microgliosis and brain inflammation found in the brain of patients with Alzheimer's disease.

In another type of amyloidosis seen in patients with Type II diabetes, the amyloidogenic protein IAPP has been shown to induce β-islet cell toxicity *in vitro*. Hence, appearance of IAPP fibrils in the pancreas of Type II diabetic patients could contribute to the loss of the β islet cells (Langerhans) and organ dysfunction.

People suffering from Alzheimer's disease develop a progressive dementia in adulthood, accompanied by three main structural changes in the brain: diffuse loss of neurons in multiple parts of the brain; accumulation of intracellular protein deposits termed neurofibrillary tangles; and accumulation of extracellular protein deposits termed amyloid or senile plaques, surrounded by misshapen nerve terminals (dystrophic neurites). A main constituent of these amyloid plaques is the amyloid-β peptide (Aβ), a 40-42 amino-acid protein that is produced through cleavage of the β-amyloid precursor protein (APP). Although symptomatic treatments exist for Alzheimer's disease, this disease cannot be prevented nor cured at this time.

The use of a vaccine to treat Alzheimer's disease is possible in principle (Schenk, D. et al., (1999) Nature 400, 173-177). Schenk et al. show that, in a transgenic mouse model of brain amyloidosis (as seen in Alzheimer's disease), immunization with Aβ peptide inhibits the formation of amyloid plaques and the associated dystrophic neurites. In that study, a vaccine using the human aggregated all-L peptide as immunogen prevented the formation of β-amyloid plaque, astrogliosis and neuritic dystrophy in vaccinated transgenic mice.

However, it is apparent that there are a number of drawbacks to using an endogenous protein as a vaccine (or a protein naturally present in the animal being vaccinated). Some of these drawbacks include:

- Possible development of autoimmune disease due to the generation of antibodies against "self" protein.
- Difficulty in eliciting an immune response due to the failure of the host immune system to recognize "self" antigens.
- Possible development of an acute inflammatory response.

SUMMARY OF THE INVENTION

The present invention relates to a stereochemically based "non-self" antigen vaccine for the prevention and/or treatment of Alzheimer's and other amyloid related diseases. One aim of the present invention is to provide a vaccine for the prevention and treatment of Alzheimer's and other amyloid related diseases, which overcomes the drawbacks associated with using naturally occurring peptides, proteins or immunogens.

In an embodiment, a vaccine is provided which is produced using a "non-self" peptide or protein synthesized from the unnatural D-configuration amino acids, to avoid the drawbacks of using "self" proteins. In accordance with the present invention, the peptides need not be aggregated to be operative or immunogenic as opposed to the prior art vaccines.

In another embodiment, there is provided a method for preventing and/or treating an amyloid-related disease in a subject, which features administering to the subject an antigenic amount of an all-D peptide which elicits production of antibodies against the all-D peptide, and elicit an immune response by the subject, therefore preventing fibrillogenesis and associated cellular toxicity, wherein the antibodies interact with at least one region of an amyloid protein, e.g., β sheet region and GAG-binding site region, immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof. These vaccines may be used in the prevention and/or treatment of amyloid related diseases, and in the manufacture of medicaments for preventing and/or treating amyloid-related diseases.

In a further embodiment of the invention, a vaccine for preventing and/or treating an amyloid-related disease in a subject comprises an antibody which interacts with amyloid proteins to prevent fibrillogenesis, wherein the antibodies are raised against an antigenic amount of an all-D peptide interacting with at least one region of an amyloid protein, e.g., β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof. These vaccines may be used in the prevention and/or treatment of amyloid related diseases, and in the manufacture of medicaments for preventing and/or treating amyloid-related diseases.

Still in a further embodiment, there is provided a method for preventing and/or treating an amyloid-related disease in a subject, which comprises administering to the subject an antigenic amount of an all-D peptide which interacts with at least one region of an amyloid protein, e.g., β sheet region and GAG-binding site region, Aβ (1-42), immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, wherein the compound elicits an immune response by the subject and therefore prevents fibrillogenesis.

In a preferred embodiment of the present invention, the compound is a compound of Formula I:

R'-(P)-R'' **(I)**,

wherein

P is an all-D peptide interacting with at least one region of an amyloid protein, e.g., β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R′ is an N-terminal substituent, e.g.:

- hydrogen;
- lower alkyl groups, e.g., acyclic or cyclic having 1 to 8 carbon atoms, without or with functional groups, e.g., carboxylate, sulfonate and phosphonate;

- aromatic groups;
- heterocyclic groups; and
- acyl groups, e.g., alkylcarbonyl, arylcarbonyl, sulfonyl and phosphonyl groups; and

R″ is a C-terminal substituent, e.g., hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

In an embodiment, R′ and R″ are identical or different, wherein alkyl or aryl group of R′ and R″ are further substituted with functionalities such as halide (e.g., F, Cl, Br, and I), hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl groups.

When the compound has an acid functional group, it can be in the form of a pharmaceutically acceptable salt or ester. When the compound has a basic functional group, it can be in the form of a pharmaceutically acceptable salt.

In a preferred embodiment of the present invention, the subject is a human being.

In yet another embodiment of the present invention, the amyloid related disease may be Alzheimer's disease.

In another embodiment of the present invention, there is provided a method for preventing and/or treating of an amyloid related disease in a subject, comprising administering to the subject an antigenic amount of a compound of Formula I:

R'-(P)-R'' **(I)**,

wherein

P is an all-D peptide interacting with at least one region of an amyloid protein, e.g., β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R′ is an N-terminal substituent selected from the group consisting of:

- hydrogen;
- lower alkyl groups, e.g., acyclic or cyclic having 1 to 8 carbon atoms, without or with functional groups, e.g., carboxylate, sulfonate and phosphonate;
- aromatic groups;

- heterocyclic groups; and
- acyl groups, e.g., alkylcarbonyl, arylcarbonyl, sulfonyl and phosphonyl groups; and

R″ is a C-terminal substituent, e.g., hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

In accordance with this method, the compound elicits an immune response by the subject, preventing fibrillogenesis.

In accordance with a preferred embodiment of the present invention, there is provided a vaccine for preventing and/or treating an amyloid-related disease in a subject, comprising an antigenic amount of an all-D peptide which interacts with at least one region of an amyloid protein, e.g., β sheet region and GAG-binding site region, Aβ (1-42, all-D) peptide, immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, wherein the compound elicits an immune response by the subject and prevents fibrillogenesis.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 illustrates the targeted sites for the antigenic fragments;

FIG. 2 illustrates the effect of 1 mg/ml of antibodies raised against D and L forms of Aβ(16-21) on fibrillogenesis;

FIG. 3 illustrates the effect of 0.5 mg/ml of antibodies raised against D and L forms of Aβ(16-21) on fibrillogenesis;

FIGs. 4A to 4C illustrate electron micrographs showing the effect of anti-D KLVFFA peptide antibodies (FIG. 4B) and anti-L KLVFFA peptide antibodies(FIG. 4C) with respect to a control (FIG. 4A) on fibrillogenesis;

FIGs. 5A to 5D illustrate the immunohistochemistry of anti-D KLVFFA on aggregated Aβ peptide in brain sections of retrosplenial cortex (FIG. 5A) and parietal cortex (FIG. 5C) and the histochemistry (Thioflavin S assay) of anti-D KLVFFA on aggregated Aβ peptide in the same brain sections of retrospinal cortex (FIG. 5B) and parietal cortex (FIG. 5D);

FIGs. 6A to 6D illustrate the immunohistochemistry of anti-L KLVFFA antibodies on aggregated Aβ peptide in brain sections of parietal cortex (FIG. 6A) and entorhinal cortex (FIG. 6C) and the histochemistry (Thioflavin S assay) of anti-L KLVFFA antibodies on aggregated Aβ peptide in the same brain sections of parietal cortex (FIG. 6B) and entorhinal cortex (FIG. 6D); and

FIG. 7 illustrates the response of rabbits to KLH-conjugated all-L and all-D KLVFFA.

DETAILED DESCRIPTION OF THE INVENTION

For the purpose of the present disclosure, the following terms are defined below.

The term "peptidomimetic" includes non-peptide compounds which mimic the structural or the functional properties of a peptide.

The term "antigenic fragment thereof" includes fragments of peptides which are capable of eliciting an immune response in a subject.

The term "amyloid related diseases" includes diseases associated with the accumulation of amyloid which can either be restricted to one organ, "localized amyloidosis", or spread to several organs, "systemic amyloidosis". Secondary amyloidosis may be associated with chronic infection (such as tuberculosis) or chronic inflammation (such as rheumatoid arthritis), including a familial form of secondary amyloidosis which is also seen in Familial Mediterranean Fever (FMF) and another type of systemic amyloidosis found in long-term hemodialysis patients. Localized forms of amyloidosis include, without limitation, diabetes type II and any related disorders thereof, neurodegenerative diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease, Alzheimer's disease, Cerebral Amyloid Angiopathy, and prion protein related disorders.

Except as otherwise expressly defined herein, the abbreviations used herein for designating the amino acids and the protective groups are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry*, 1972, 11:1726-1732).

The Aβ(16-21) site is known to play an important role in initiating the harmful process of Aβ peptide amyloidogenesis. It is also known that when these peptides are made from D-amino acids, they retain their ability to interact with the natural all-L-homologous sequence, thereby preventing amyloidogenesis.

Other amyloid proteins which may be used in the present invention include, without limitation, IAPP, β2-microglubeline, amyloid A protein, and prion-related proteins.

The vaccine of the present invention, prepared from all-D-Aβ(16-21), D-Aβ(10-16), D-Aβ(1-40), D-Aβ(1-42) or the C-terminal region of D-Aβ(1-42), is believed to elicit an immune response in the host or in producing antibodies that recognize the naturally occurring target. As used herein, "all-D" includes peptides having ≥ 75%, ≥ 80%, ≥ 85%, ≥ 90%, ≥ 95%, and 100% D-configuration amino acids. Also, the vaccine of the present invention does not present the drawbacks of using "self" proteins and does not need to be aggregated to induce an immune response. For example, the antibodies raised against the all-D-Aβ(16-21) peptide can be expected to recognize the all-L-Aβ(16-21) peptide sequence.

The elicited antibodies present in the host having received the vaccine of the present invention bind at the Aβ(16-21) site or other sites such as the C-terminal region of Aβ and have the same or even greater ability to prevent amyloidogenesis as do the short peptides themselves. The vaccine of the present invention causes the generation of effective antiamyloidogenic antibodies in the vaccinated host.

A suggested immunization procedure is as follows:

a) prepare a vaccine from an all-D peptide having a sequence substantially the same as that of a naturally occurring β amyloid peptide, namely Aβ (all-L). The all-D peptide includes a full length Aβ (1-42, all-D), a peptide derived from an immunogenic fragment of Aβ (1-42, all-D), and a related peptidomimetic;

b) immunize a host with the vaccine to generate an antibody in the host with a binding site capable of preventing fibrillogenesis.

Suitable pharmaceutically acceptable carriers include, without limitation, any non-immunogenic pharmaceutical adjuvants suitable for oral, parenteral, intravascular (IV), intraarterial (IA), intramuscular (IM), and subcutaneous (SC) administration routes, such as phosphate buffer saline (PBS).

The pharmaceutical carriers may contain a vehicle, which carries antigens to antigen-presenting cells. Examples of vehicles are liposomes, immune-stimulating complexes, microfluidized squalene-in-water emulsions, microspheres which may be composed of poly(lactic/glycolic) acid (PLGA). Particulates of defined dimensions (<5 micron) include, without limitation, oil-in-water microemulsion (MF59) and polymeric microparticules.

The carriers of the present invention may also include chemical and genetic adjuvants to augment immune responses or to increase the antigenicity of antigenic immunogens. These adjuvants exert their immunomodulatory properties through several mechanisms such as lymphoid cells recruitment, cytokine induction, and the facilitation of DNA entry into cells. Cytokine adjuvants include, without limitation, granulocyte-macrophage colony-stimulating factor, interleukin-12, GM-CSF, synthetic muramyl dipeptide analog or monophosphoryl lipid A. Other chemical adjuvants include, without limitation, lactic acid bacteria, $Al(OH)_3$, muramyl dipeptides and saponins.

The peptide may be coupled to a carrier that will modulate the half-life of the circulating peptide. This will allow the control on the period of protection. The peptide-carrier may also be emulsified in an adjuvant and administrated by usual immunization route.

The vaccine of the present invention will, for the most part, be administered parenterally, such as intravascularly (IV), intraarterially (IA), intramuscularly (IM), subcutaneously (SC), or the like. In some instances, administration may be oral, nasal, rectal, transdermal or aerosol, where the nature of the vaccine allows for transfer to the vascular system. Usually a single injection will be employed although more than one injection may be used, if desired. The vaccine may be administered by any convenient means, including syringe, trocar, catheter, or the like. Preferably, the administration will be intravascularly, where the site of introduction is not critical to this invention, preferably at a site where there is rapid blood flow, e.g., intravenously, peripheral or central vein. Other routes may find use where the administration is coupled with slow release techniques or a protective matrix.

The use of the vaccine of the present invention in preventing and/or treating Alzheimer's disease and other amyloid related diseases can be validated by raising antibodies against the corresponding all-D peptide and testing them to see if they can effectively inhibit or prevent the fibrillogenesis of the natural amyloid peptide (all-L).

The compounds used to prepare vaccines in accordance with the present invention have the common structure of Formula I:

$$R'\text{-}(P)\text{-}R'' \quad \textbf{(I)},$$

wherein

P is an all-D peptide interacting with at least one region of an amyloid protein, e.g., β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R′ is an N-terminal substituent selected from the group consisting of:

- hydrogen;
- lower alkyl groups, e.g., acyclic or cyclic having 1 to 8 carbon atoms, without or with functional groups, e.g., carboxylate, sulfonate and phosphonate;
- aromatic groups;
- heterocyclic groups; and
- acyl groups, e.g., alkylcarbonyl, arylcarbonyl, sulfonyl and phosphonyl groups; and

R″ is a C-terminal substituent, e.g., hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

R′ and R″ may be identical or different; the alkyl or aryl group of R′ and R″ may further be substituted with organic functionalities selected from the group of halides (F, Cl, Br, and I), hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl, and the like.

Where a functional group is an acid, its pharmaceutically acceptable salt or ester is in the scope of this invention. Where a functional group is a base, its pharmaceutically acceptable salt is in the scope of this invention.

In one embodiment, the preferred compounds are selected from the full-length peptide, Aβ (1-42, all-D), and its lower homologues consisting of Aβ (1-40, all-D), Aβ (1-35, all-D), and Aβ (1-28, all-D).

In another embodiment, the preferred compounds are selected from a group of short peptides, e.g., Aβ (1-7, all-D), Aβ (10-16, all-D), Aβ (16-21, all-D), Aβ (36-42, all-D). The peptides can be shortened further by removing one or more residues from either end or both ends.

The preferred compounds may also be all-D peptides derived from the peptides above by substitution of one or more residues in the naturally occurring sequence. In another embodiment, the preferred compounds are peptidomimetics of the above-said peptides.

In a further embodiment, the preferred compounds may be coupled with a carrier that will modulate the biodistribution, immunogenic property and the half-life of the compounds.

The following are exemplary compounds for preparing vaccines for preventing or treating Alzheimer's disease and other amyloid related diseases:

1 Aβ (1-42, all-D)
2 Aβ (1-40, all-D)
3 Aβ (1-35, all-D)
4 Aβ (1-28, all-D)
5 Aβ (1-7, all-D)
6 Aβ (10-16, all-D)
7 Aβ (16-21, all-D)
8 Aβ (36-42, all-D)
9 Lys-Ile-Val-Phe-Phe-Ala (all-D)
10 Lys-Lys-Leu-Val-Phe-Phe-Ala (all-D)

11 Lys-Phe-Val-Phe-Phe-Ala (all-D)

12 Ala-Phe-Phe-Val-Leu-Lys (all-D)

13 Lys-Leu-Val-Phe (all-D)

14 Lys-Ala-Val-Phe-Phe-Ala (all-D)

15 Lys-Leu-Val-Phe-Phe (all-D)

16 Lys-Val-Val-Phe-Phe-Ala (all-D)

17 Lys-Ile-Val-Phe-Phe-Ala-NH$_2$ (all-D)

18 Lys-Leu-Val-Phe-Phe-Ala-NH$_2$ (all-D)

19 Lys-Phe-Val-Phe-Phe-Ala-NH$_2$ (all-D)

20 Ala-Phe-Phe-Val-Leu-Lys-NH$_2$ (all-D)

21 Lys-Leu-Val-Phe-NH$_2$ (all-D)

22 Lys-Ala-Val-Phe-Phe-Ala-NH$_2$ (all-D)

23 Lys-Leu-Val-Phe-Phe-NH$_2$ (all-D)

24 Lys-Val-Val-Phe-Phe-Ala-NH$_2$ (all-D)

25 Lys-Leu-Val-Phe-Phe-Ala-Gln (all-D)

26 Lys-Leu-Val-Phe-Phe-Ala-Gln-NH$_2$ (all-D)

27 His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Gln (all-D)

28 Asp-Asp-Asp (all-D)

29 Lys-Val-Asp-Asp-Gln-Asp (all-D)

30 His-His-Gln-Lys (all-D)

31 Phe-Phe-NH-CH$_2$CH$_2$SO$_3$H (all-D)

32 Phe-Phe-NH-CH$_2$CH$_2$CH$_2$SO$_3$H (all-D)

33 Phe-Phe-NH-CH$_2$CH$_2$CH$_2$CH$_2$SO$_3$H (all-D)

34 Phe-Tyr-NH-CH$_2$CH$_2$SO$_3$H (all-D)

35 Phe-Tyr-NH-CH$_2$CH$_2$CH$_2$SO$_3$H (all-D)

36 Phe-Tyr-NH-CH$_2$CH$_2$CH$_2$CH$_2$SO$_3$H (all-D)

37 HO$_3$SCH$_2$CH$_2$-Phe-Phe (all-D)

38 HO$_3$SCH$_2$CH$_2$CH$_2$-Phe-Phe (all-D)

39 HO$_3$SCH$_2$CH$_2$CH$_2$CH$_2$-Phe-Phe (all-D)

40 HO$_3$SCH$_2$CH$_2$-Phe-Tyr (all-D)

41 HO$_3$SCH$_2$CH$_2$CH$_2$-Phe-Tyr (all-D)

42 HO$_3$SCH$_2$CH$_2$CH$_2$CH$_2$-Phe-Tyr (all-D)

43 HO$_3$SCH$_2$CH$_2$-Leu-Val-Phe-Phe-Ala (all-D)

44 $HO_3SCH_2CH_2CH_2$-Leu-Val-Phe-Phe-Ala (all-D)
45 $HO_3SCH_2CH_2CH_2CH_2$-Leu-Val-Phe-Phe-Ala (all-D)
46 Leu-Val-Phe-Phe-Ala-NH-$CH_2CH_2SO_3H$ (all-D)
47 Leu-Val-Phe-Phe-Ala-NH-$CH_2CH_2CH_2SO_3H$ (all-D)
48 Leu-Val-Phe-Phe-Ala-NH-$CH_2CH_2CH_2CH_2SO_3H$ (all-D).

The compounds listed above may be modified by removing or inserting one or more amino acid residues, or by substituting one or more amino acid residues with other amino acid or non-amino acid fragments.

The following are exemplary compounds derived from compound 18 (all-D KLVFFA-NH_2) by substituting one or two amino acid residue(s) with other amino acids.

49 Lys-Leu-Val-Trp-Phe-Ala-NH_2(all-D)
50 Lys-Leu-Val-Phe-Trp-Ala- NH_2 (all-D)
51 Lys-Leu-Val-Trp-Trp-Ala- NH_2 (all-D)
52 Lys-Leu-Val-Tyr-Phe-Ala- NH_2 (all-D)
53 Lys-Leu-Val-Phe-Tyr-Ala- NH_2 (all-D)
54 Lys-Leu-Val-Tyr-Tyr-Ala- NH_2 (all-D)
55 Lys-Leu-Val-Thi-Phe-Ala- NH_2 (all-D)
56 Lys-Leu-Val-Phe-Thi-Ala- NH_2 (all-D)
57 Lys-Leu-Val-Thi-Thi-Ala- NH_2 (all-D)
58 Lys-Leu-Val-Cha-Phe-Ala- NH_2 (all-D)
59 Lys-Leu-Val-Phe-Cha-Ala- NH_2 (all-D)
60 Lys-Leu-Val-Cha-Cha-Ala- NH_2 (all-D)
61 Lys-Leu-Val-Pgly-Phe-Ala- NH_2 (all-D)
62 Lys-Leu-Val-Phe-Pgly-Ala- NH_2 (all-D)
63 Lys-Leu-Val-Pgly-Pgly-Ala- NH_2 (all-D).

For the above compounds, the terms Thi, Cha and Pgly are intended to mean thienylalanine, cyclohexylalanine and phenylglycine, respectively.

Rabbits were immunized with all-D or all-L KLVFFA. Results of the antibody titers obtained are shown in FIG. 7. As seen in FIG. 7, the vaccine of the present invention causes production of antibodies.

The present invention encompasses various types of immune responses triggered using the vaccine of the present invention, e.g., amyloid therapies using the vaccine approach.

In accordance with the present invention, there is also provided a vaccine which triggers a preferential TH-2 response or a TH-1 response, according to the type of immunization used. By inducing a TH-2 response, anti-inflammatory cytokine production such as IL-4, Il-10 and TGF-β, as well as the production of IgG 1 and IgG 2b antibody classes, are favored. Such type of response would be preferred, as a major inflammatory response in the brain of the patients with AD would be avoided. On the other hand, with a preferred TH-1 response, a pro-inflammatory response with a production of inflammatory cytokines such as IL-1, Il-6, TNF and IFN gamma would be favored. This type of response would more likely trigger activation of the macrophage population. These macrophages would then phagocytose any particulate deposits (such as plaques) via a complement-activated process as well as via antibody-mediated process. This approach would be beneficial to clear already organized senile plaques and prevent the formation of new fibrillary deposits.

Both approaches (i.e. TH-1 and TH-2) are of value. The antigen used could be the peptides which contain regions responsible for cellular adherence, i.e., region 10-16, regions responsible for the GAG binding site, i.e., 13-16, regions responsible for the β sheet 16-21 or regions for 40-42. These peptides could be presented in such a way that either a preferential TH-1 or TH-2 response is obtained, depending on the type of adjuvant used, or depending on the route of administration of the vaccine. For example, a mucosal immunization via nasal administration is possible, since it is known that such a route of administration would favor a TH-2 response.

The present invention will be more readily understood by referring to the following examples, which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

An *in vitro* validation procedure to test the effectiveness of all-D peptide vaccines derived from fibrillogenic proteins was performed in rabbits or mice to demonstrate that antibodies can be raised against Aβ 16-21 (all-D) (see FIG. 7). The antibodies produced were tested to prove that they effectively prevent the fibrillogenesis of natural Aβ(1-40, all-L) *in vitro*. Standard assays for fibrillogenesis were used to evaluate activity, such as those based on Thioflavine T, circular dichroism and solubility.

This approach could also be used to establish which areas of the Aβ peptide are most effective when used in the form of all-D peptides to prepare antifibrillogenic vaccines. One way this could be performed is as follows:

a) rabbits or mice are immunized with a series of overlapping all-D peptides generated from the Aβ(1-42) sequence, e.g., Aβ(1-6), Aβ(2-8), Aβ(4-10), etc.

b) antisera are prepared from the immunized rabbits or mice.

c) these antisera are tested to see which parts of the Aβ sequence produce antisera which most effectively prevents fibrillogenesis in the standard assays for fibrillogenesis mentioned above.

EXAMPLE II

Effect of Antibodies Against D- and L-Aβ(16-21) Peptide Vaccine on Fibrillogenesis

A validation procedure to test anti-fibrillogenic activity of antibodies raised against D- and L- Aβ(16-21) peptide was performed.

Rabbits were immunized with D- or L-Aβ (16-21) peptide. Antibodies raised were tested for their antifibrillogenic activities by ThT assay and by electron microscopy (EM).

Antibodies raised against the D- and L- forms of KLVFFA were capable of blocking the fibrillogenesis process as seen either by the Thioflavin T assay (ThT) (FIGs. 2 and 3) and by EM (FIGs. 4A to 4C). In the ThT assay, fibril formation is monitored by the increase in fluorescence with time. As seen in the Figures, the antibodies were capable of inhibiting such an increase in fluorescence, proving that these antibodies were inhibiting fibrillogenesis.

As can be seen in these figures (FIGs. 2 to 4), antibodies raised against the D-peptide have a better anti-fibrillogenic activity than anti-L antibodies.

These results were also confirmed by EM (FIGs. 4A to 4C) where both anti-D and anti-L KLVFFA peptide blocked the fibril formation when compared to control (FIG. 4A). Moreover, again the anti-D peptide has a greater anti-fibrillogenic activity (FIG. 4B) than the anti-L peptide (FIG. 4C). This goes along with the ThT assay where the decrease in fluorescence was greater with the anti-D peptide antibody than with the anti-L peptide antibody.

EXAMPLE III

Antibody Binding Assay

Brain sections were stained with antibodies raised against KLVFFA peptide (D and L forms). As seen in FIGs 5A to 5D and 6A to 6D, the antibodies were not capable of binding to aggregated (ThioS positive) Aβ. It can be seen from both sets of figures, which were stained for both plaques (ThioS) and anti-peptides that the antibodies are recognizing Aβ at the surface of the cells but are not capable of binding to plaques. These results show that the anti-KLVFFA peptide antibody is recognizing the non-fibrillary Aβ but does not bind to aggregated Aβ. There was no difference between the anti-D and anti-L peptide antibodies in this assay.

These results clearly prove that the antibody recognizes only the non-aggregated form and blocks the fibrillogenesis. By having such activity, the vaccine of the present invention 1) prevents Aβ from organizing itself into a fibril and 2) prevents an inflammatory response being triggered by such an antibody binding to an insoluble form, since the antibody is not able to bind to aggregated Aβ.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

CLAIMS

What is claimed is:

1. A method for preventing and/or treating an amyloid-related disease in a subject, comprising administering to a subject an antigenic amount of an all-D peptide which elicits production of antibodies against said all-D peptide, and elicit an immune response by said subject, therefore preventing fibrillogenesis and associated cellular toxicity, wherein said antibodies and/or immune cells interact with at least one region of an amyloid protein selected from the group consisting of β sheet region and GAG-binding site region, immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof,.

2. The method of claim 1, wherein said compound is a compound of Formula I:

R'-(P)-R'' **(I)**,

wherein

P is an all-D peptide interacting with at least one region of an amyloid protein selected from the group consisting of β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R′ is an N-terminal substituent selected from the group consisting of:

hydrogen;
lower alkyl groups selected from the group consisting of acyclic or cyclic having 1 to 8 carbon atoms;
aromatic groups;
heterocyclic groups; and
acyl groups; and

R″ is a C-terminal substituent selected from the group consisting of hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

3. The method of claim 2, wherein said alkyl or aryl group of R′ and R″ is further substituted with halide; hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl groups.

4. The method of claim 2, wherein said compound further comprises an acid functional group, a pharmaceutically acceptable salt or ester form thereof; or a base functional group or pharmaceutically acceptable salt form thereof.

5. The method of claim 2, wherein said compound is selected from the group consisting of compounds 1 to 48.

6. The method of claim 5, wherein said compound is modified by removing or inserting one or more amino acid residues, or by substituting one or more amino acid residues with other amino acid or non-amino acid fragment.

7. The method of claim 6, wherein said compound is selected from the group consisting of compounds 49 to 63.

8. The method of claim 1, wherein said subject is a human being.

9. The method of claim 1, wherein said disease is Alzheimer's disease.

10. A vaccine for preventing and/or treating an amyloid-related disease in a subject, comprising an antibody raised against an antigenic amount of an all-D peptide which interacts with at least one region of an amyloid protein selected from the group consisting of β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, wherein said antibody interacts with amyloid proteins and therefore prevents fibrillogenesis.

11. The vaccine of claim 10, wherein said compound is a compound of Formula I:

R'-(P)-R'' **(I)**,

wherein

P is an all-D peptide interacting with at least one region of an amyloid protein selected from the group consisting of β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R′ is an N-terminal substituent selected from the group consisting of:

hydrogen;
lower alkyl groups selected from the group consisting of acyclic or cyclic having 1 to 8 carbon atoms;
aromatic groups;
heterocyclic groups; and
acyl groups; and

R″ is a C-terminal substituent selected from the group consisting of hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

12. The vaccine of claim 10, wherein said alkyl or aryl group of R′ and R″ are further substituted with halide; hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl groups.

13. The vaccine of claim 11, wherein said compound further comprises an acid functional group, a pharmaceutically acceptable salt or ester form thereof; or a base functional group or pharmaceutically acceptable salt form thereof.

14. The vaccine of claim 10, wherein said compound is selected from the group consisting of compounds 1 to 48.

15. The vaccine of claim 11, wherein said compound is modified by removing or inserting one or more amino acid residues, or by substituting one or more amino acid residues with other amino acid or non-amino acid fragment.

16. The method of claim 15, wherein said compound is selected from the group consisting of compounds 49 to 63.

17. The vaccine of claim 10, wherein said subject is a human being.

18. The vaccine of claim 10, wherein said disease is Alzheimer's disease.

19. Use of a vaccine as defined in claim 10, 11, 12, 13, 14, 15, 16, 17 or 18 for preventing and/or treating an amyloid-related disease.

20. Use of a vaccine as defined in claim 10, 11, 12, 13, 14, 15, 16, 17 or 18 for the manufacture of a medicament for preventing and/or treating an amyloid-related disease.

21. A method for preventing and/or treating an amyloid-related disease in a subject, comprising administering to said subject an antigenic amount of an all-D peptide which interacts with at least one region of an amyloid protein selected from the group consisting of β sheet region and GAG-binding site region, immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, wherein said compound elicits an immune response by said subject and therefore prevents fibrillogenesis.

22. The method of claim 21, wherein said compound is a compound of Formula I:

$$R'\text{-}(P)\text{-}R'' \qquad \textbf{(I)},$$

wherein

P is an all-D peptide interacting with at least one region of an amyloid protein selected from the group consisting of β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R′ is an N-terminal substituent selected from the group consisting of:

hydrogen;
lower alkyl groups selected from the group consisting of acyclic or cyclic having 1 to 8 carbon atoms;
aromatic groups;
heterocyclic groups; and
acyl groups; and

R″ is a C-terminal substituent selected from the group consisting of hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

23. The method of claim 22, wherein said alkyl or aryl group of R′ and R″ are further substituted with halide; hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl groups.

24. The method of claim 22, wherein said compound further comprises an acid functional group, a pharmaceutically acceptable salt or ester form thereof; or a base functional group or a pharmaceutically acceptable salt form thereof.

25. The method of claim 22, wherein said compound is selected from the group consisting of compounds 1 to 48.

26. The method of claim 25, wherein said compound is modified by removing or inserting one or more amino acid residues, or by substituting one or more amino acid residues with other amino acid or non-amino acid fragment.

27. The method of claim 26, wherein said compound is selected from the group consisting of compounds 49 to 63.

28. The method of claim 21, wherein said subject is a human being.

29. The method of claim 21, wherein said disease is Alzheimer's disease.

30. A method for preventing and/or treating of an amyloid related disease in a subject, which comprises administering to the said subject an antigenic amount of a compound of Formula I:

$$R'\text{-}(P)\text{-}R'' \qquad \textbf{(I)},$$

wherein

P is an all-D peptide interacting with at least one region of an amyloid protein selected from the group consisting of β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R′ is an N-terminal substituent selected from the group consisting of:

hydrogen;
lower alkyl groups selected from the group consisting of acyclic or cyclic having 1 to 8 carbon atoms;
aromatic groups;
heterocyclic groups; and
acyl groups; and

R″ is a C-terminal substituent selected from the group consisting of hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

31. The method of claim 30, wherein said compound elicits an immune response by said subject and therefore prevents fibrillogenesis.

32. The method of claim 30, wherein the alkyl or aryl group of R′ and R″ are further substituted with halide; hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl groups.

33. The method of claim 32, wherein said compound has an acid functional group, a pharmaceutically acceptable salt or ester form thereof; or a base functional group or pharmaceutically acceptable salt thereof.

34. The method of claim 30, wherein said compound is selected from the group consisting of compounds 1 to 48.

35. The method of claim 34, wherein said compound is modified by removing or inserting one or more amino acid residues, or by substituting one or more amino acid residues with other amino acid or non-amino acid fragment.

36. The method of claim 35, wherein said compound is selected from the group consisting of compounds 49 to 63.

37. The method of claim 30, wherein said subject is a human being.

38. The method of claim 30, wherein said disease is Alzheimer's disease.

39. A vaccine for preventing and/or treating an amyloid-related disease in a subject, comprising an antigenic amount of an all-D peptide interacting with at least one region of an amyloid protein selected from the group consisting of β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, protein conjugates thereof, immunogenic derivative peptides thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof, wherein said

compound elicits an immune response by said subject and therefore prevents fibrillogenesis.

40. The vaccine of claim 39, wherein said compound is a compound of Formula I:

R'-(P)-R'' **(I)**,

wherein

P is an all-D peptide interacting with at least one region of an amyloid protein selected from the group consisting of β sheet region and GAG-binding site region, Aβ (1-42, all-D), immunogenic fragments thereof, immunogenic derivatives thereof, protein conjugates thereof, immunogenic peptides thereof, and immunogenic peptidomimetics thereof;

R′ is an N-terminal substituent selected from the group consisting of:

hydrogen;
lower alkyl groups selected from the group consisting of acyclic or cyclic having 1 to 8 carbon atoms;
aromatic groups;
heterocyclic groups; and
acyl groups; and

R″ is a C-terminal substituent selected from the group consisting of hydroxy, alkoxy, aryloxy, unsubstituted or substituted amino groups.

41. The vaccine of claim 40, wherein said alkyl or aryl group of R′ and R″ is further substituted with halide; hydroxyl, alkoxyl, aryloxyl, hydroxycarbonyl, alkoxylcarbonyl, aryloxycarbonyl, carbamyl, unsubstituted or substituted amino, sulfo or alkyloxysulfonyl, phosphono or alkoxyphosphonyl groups.

42. The vaccine of claim 40, wherein said compound has an acid functional group, a pharmaceutically acceptable salt or ester form thereof; or said compound has a base functional group or pharmaceutically acceptable salt form thereof.

43. The vaccine of claim 39, wherein said compound is selected from the group consisting of compounds 1 to 48.

44. The vaccine of claim 40, wherein said compound is modified by removing or inserting one or more amino acid residues, or by substituting one or more amino acid residues with other amino acid or non-amino acid fragment.

45. The method of claim 44, wherein said compound is selected from the group consisting of compounds 49 to 63.

46. The vaccine of claim 39, wherein said subject is a human being.

47. The vaccine of claim 39, wherein said disease is Alzheimer's disease.

48. Use of a vaccine as defined in claim 39, 40, 41, 42, 43, 44, 45, 46 or 47 for preventing and/or treating an amyloid-related disease.

49. Use of a vaccine as defined in claim 39, 40, 41, 42, 43, 44, 45, 46 or 47 for the manufacture of a medicament for preventing and/or treating an amyloid-related disease.

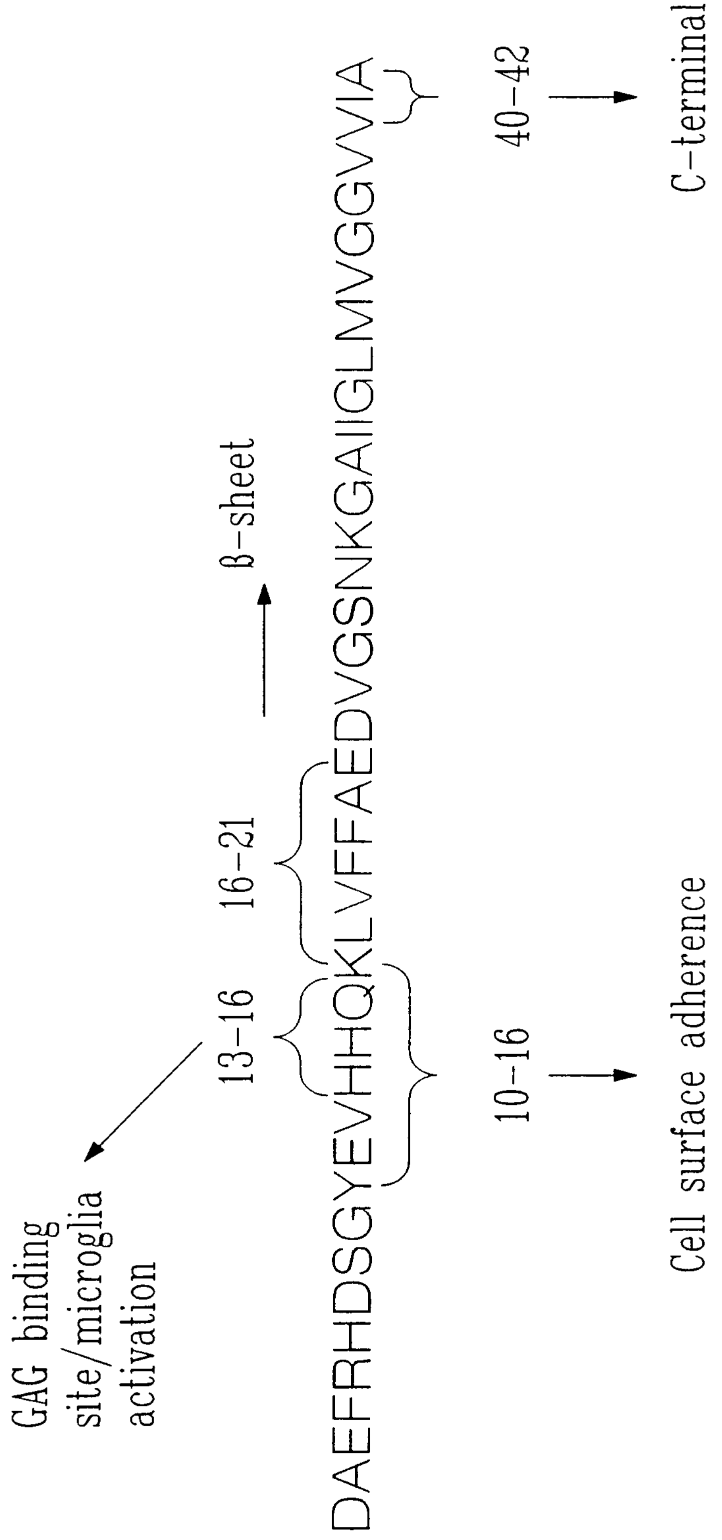

GAG binding site/microglia activation
13–16
16–21
β-sheet
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
10–16
40–42
Cell surface adherence
C–terminal

FIG. 1

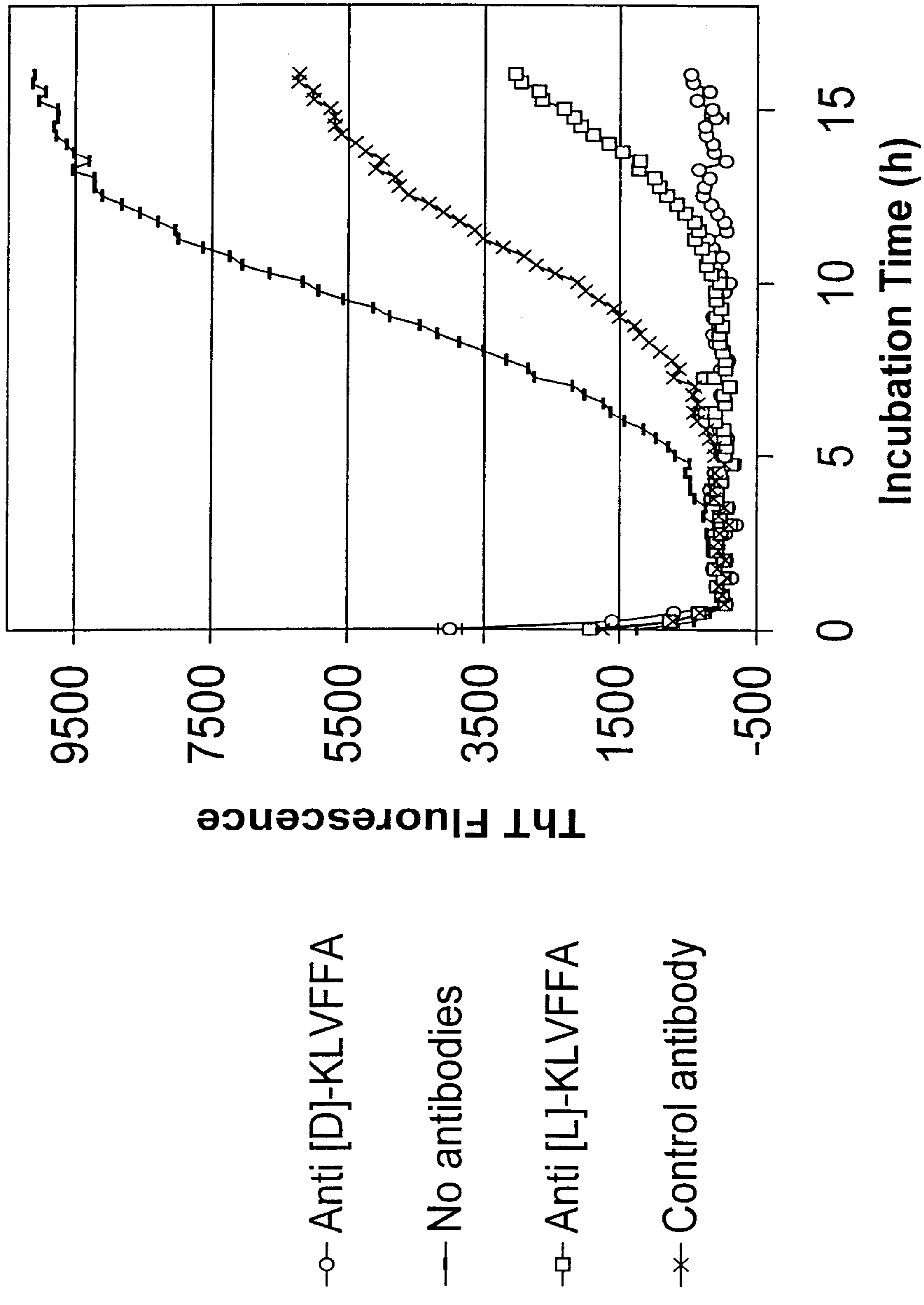

ThT Fluorescence
9500
7500
5500
3500
1500
-500
0
5
10
15
Incubation Time (h)
Anti [D]-KLVFFA
No antibodies
Anti [L]-KLVFFA
Control antibody

FIG. 2

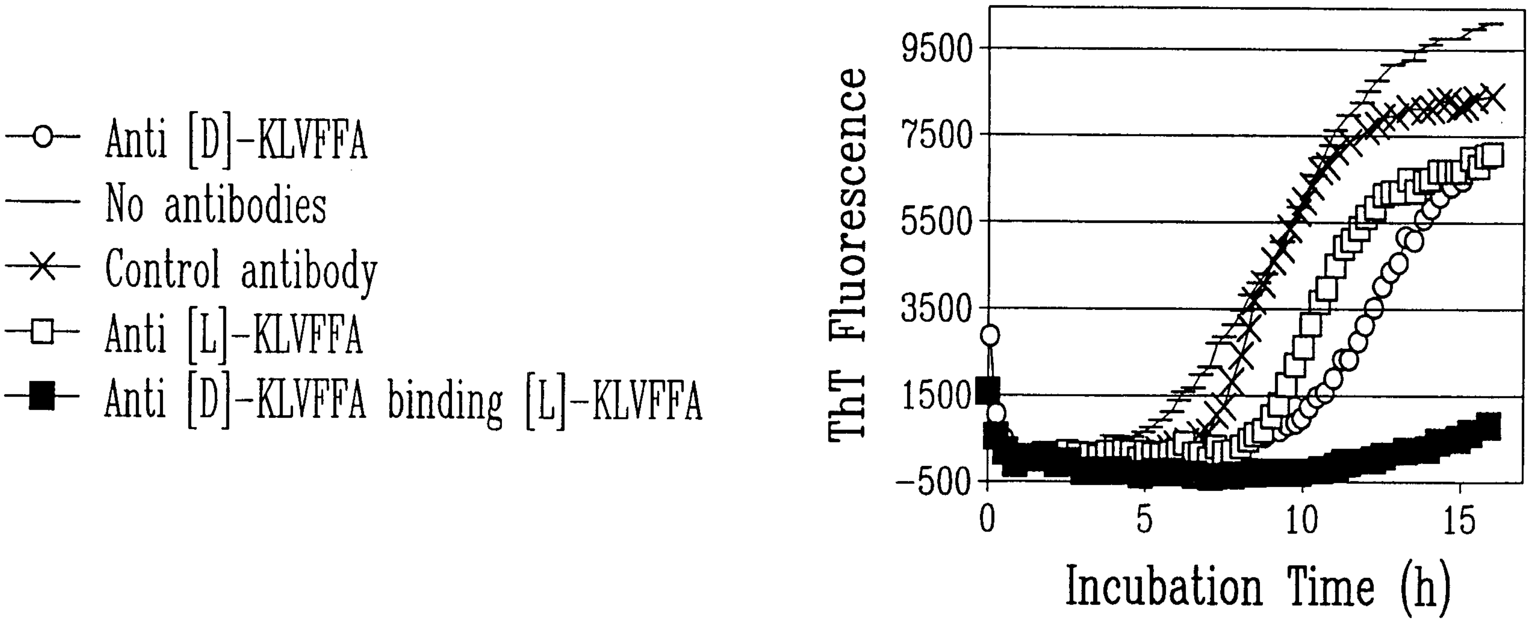

Anti [D]-KLVFFA
No antibodies
Control antibody
Anti [L]-KLVFFA
Anti [D]-KLVFFA binding [L]-KLVFFA
ThT Fluorescence
9500
7500
5500
3500
1500
-500
0
5
10
15
Incubation Time (h)

FIG. 3

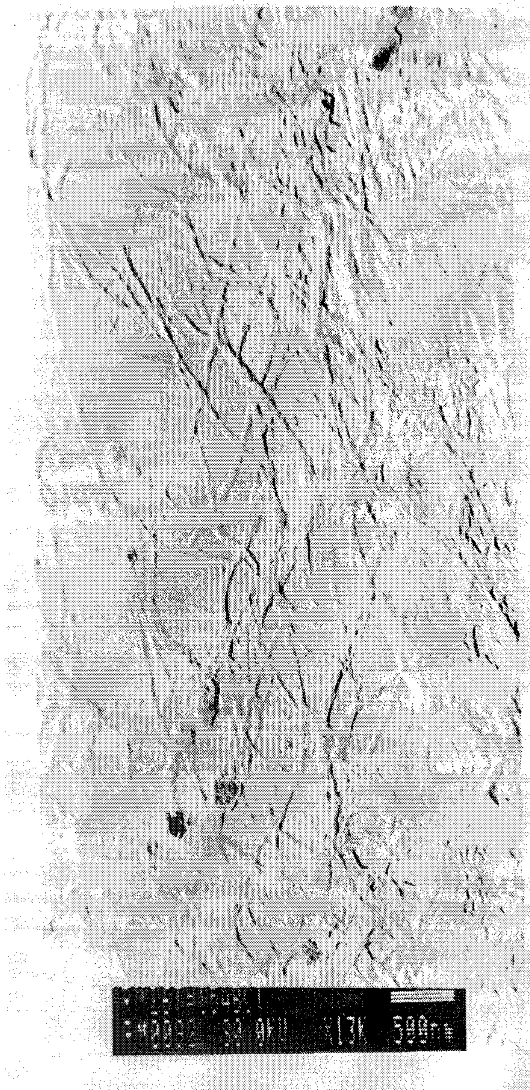


FIG-4A

FIG-4B

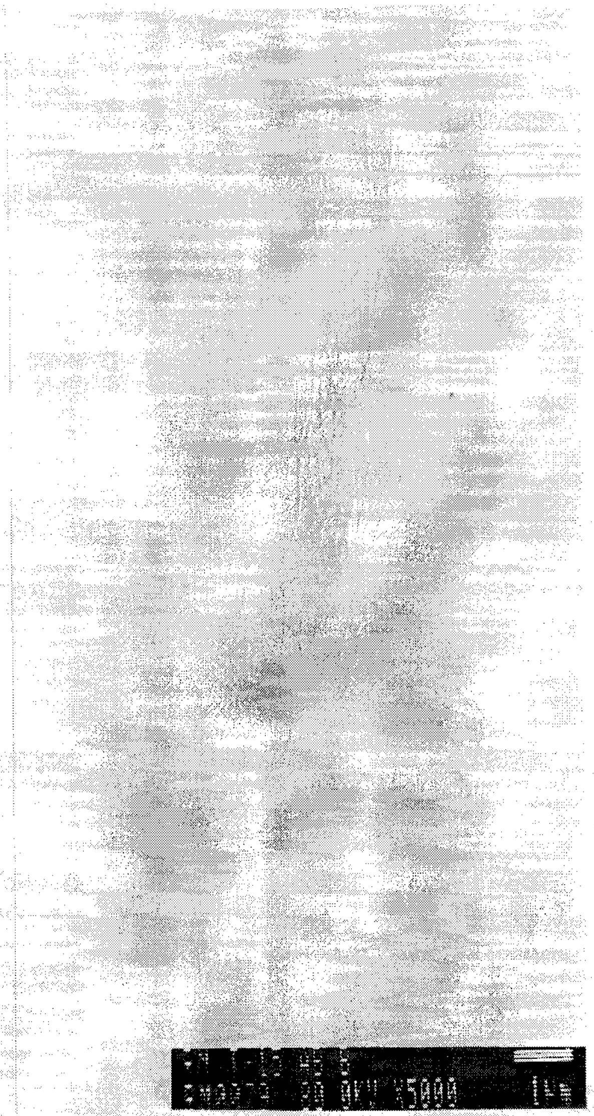


FIG-4C

IMMUNOHISTOCHEMISTRY FOR A 151

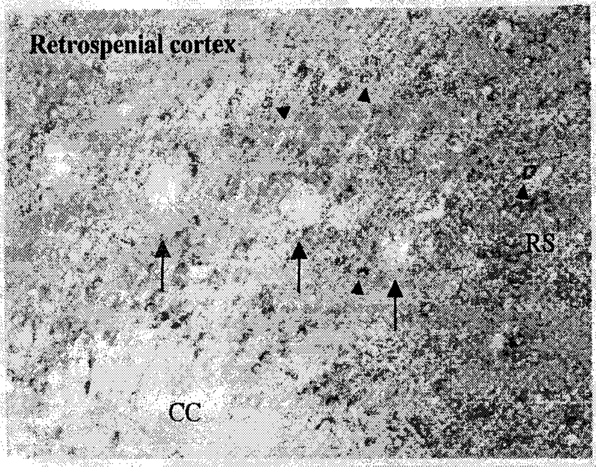


FIG. 5A

HISTOCHEMISTRY FOR THIOFLAVIN S

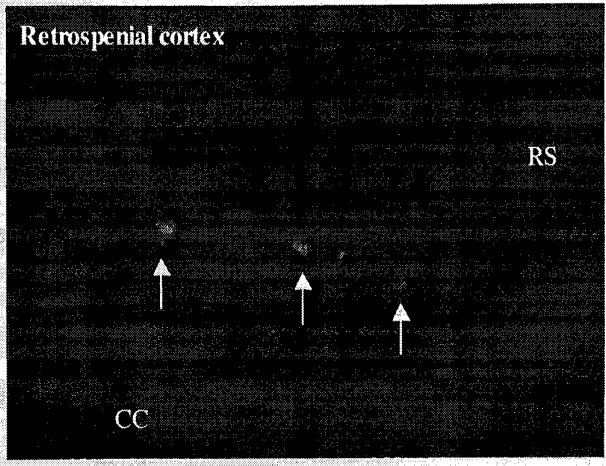


FIG. 5B

IMMUNOHISTOCHEMISTRY FOR A 151

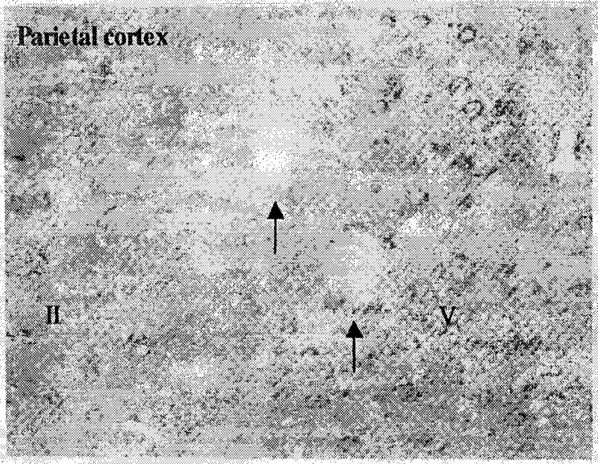


FIG. 5C

HISTOCHEMISTRY FOR THIOFLAVIN S

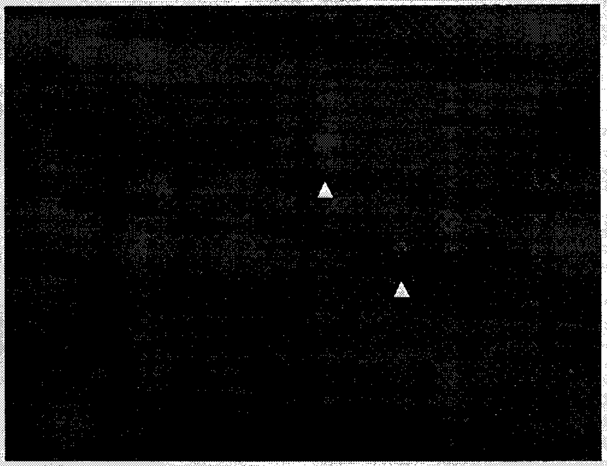


FIG. 5D

IMMUNOHISTOCHEMISTRY FOR C151

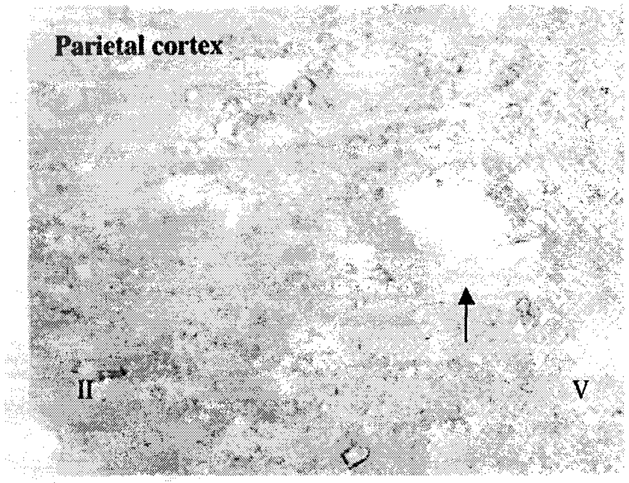


Fig. 6A

HISTOCHEMISTRY FOR THIOFLAVIN S

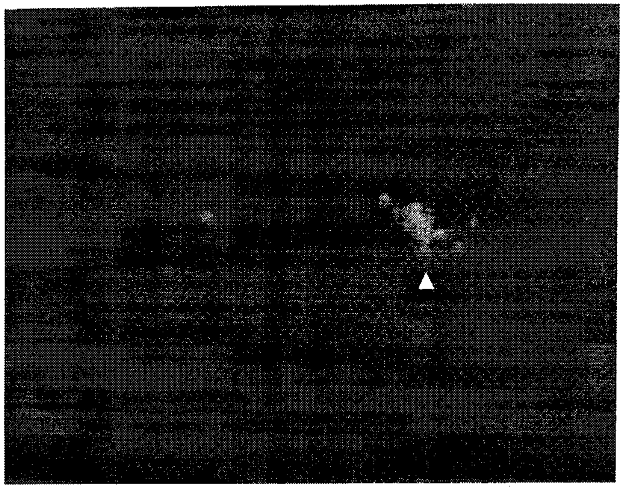


Fig. 6B

IMMUNOHISTOCHEMISTRY FOR C151

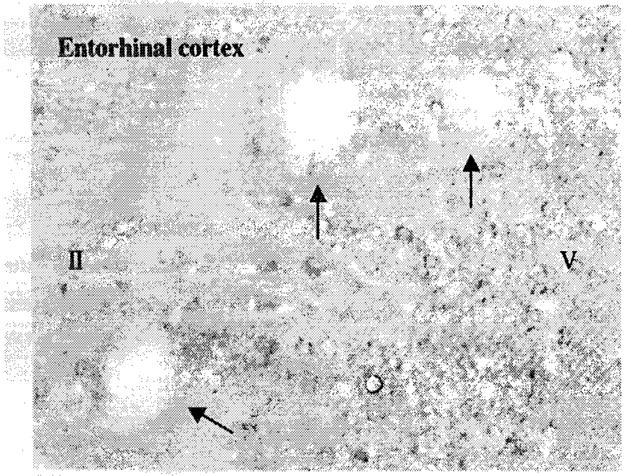


Fig. 6C

HISTOCHEMISTRY FOR THIOFLAVIN S

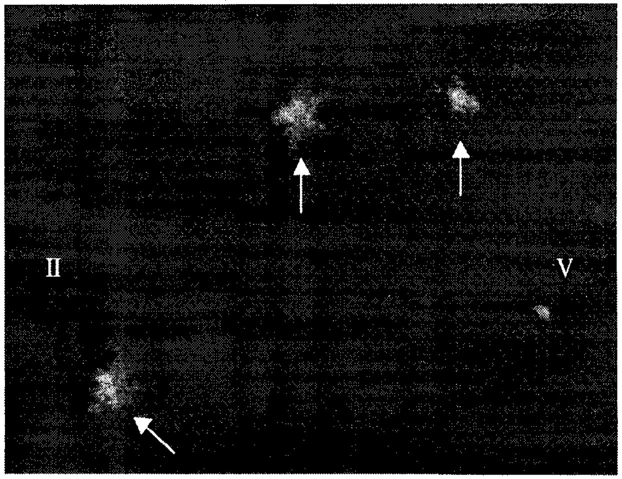


Fig. 6D

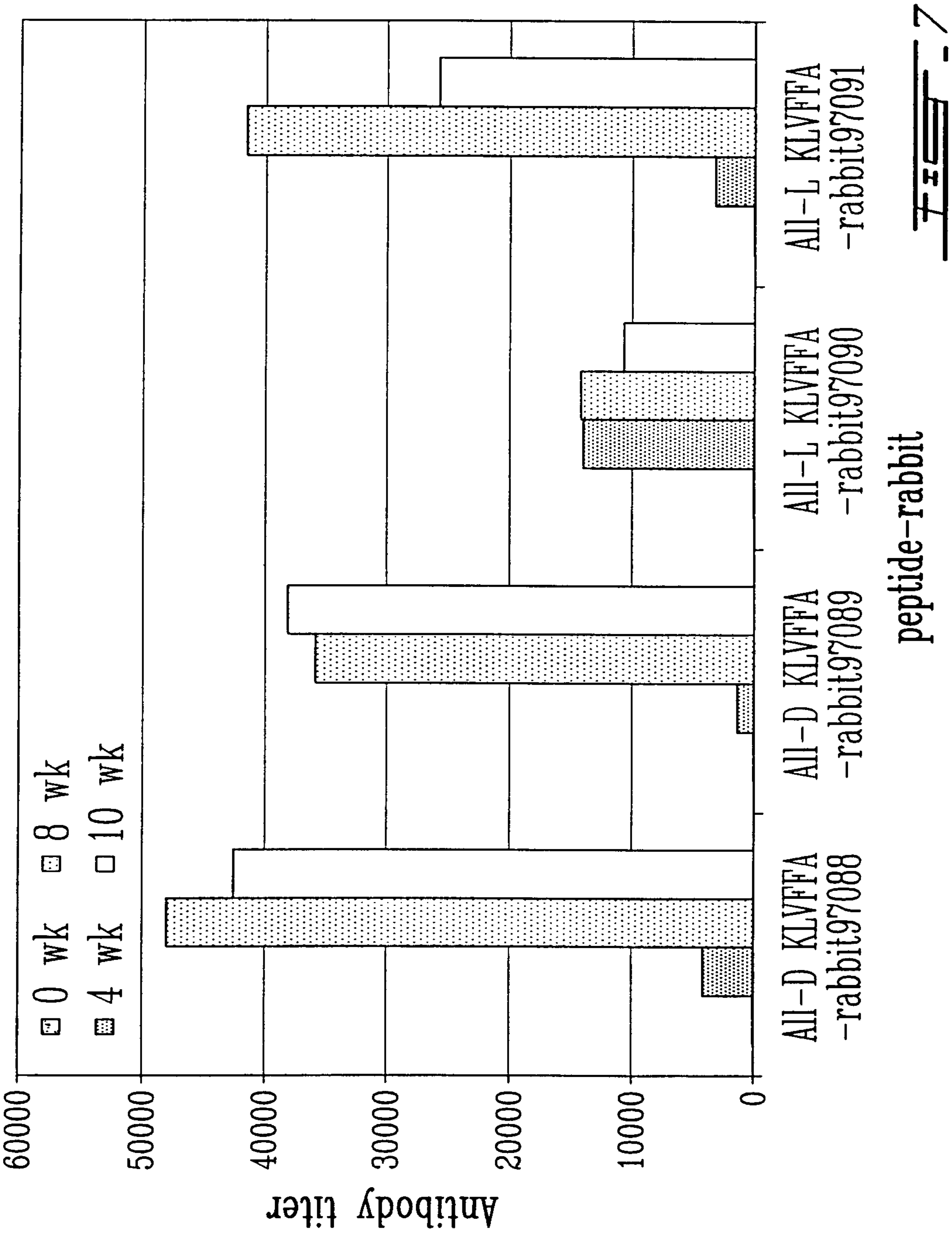

▨ 0 wk
▨ 4 wk
▨ 8 wk
☐ 10 wk
Antibody titer
60000
50000
40000
30000
20000
10000
0
All-D KLVFFA -rabbit97088
All-D KLVFFA -rabbit97089
All-L KLVFFA -rabbit97090
All-L KLVFFA -rabbit97091
peptide-rabbit

FIG. 7